1981

# On the Biosynthesis of Irregular Terpenes: Mechanistic Studies Employing the Thiamine Thiazole as an Isoprenoid Analogue.

Steven J. Jungk
*Louisiana State University and Agricultural & Mechanical College*

JUNGK, STEVEN J.

# ON THE BIOSYNTHESIS OF IRREGULAR TERPENES: MECHANISTIC STUDIES EMPLOYING THE THIAMINE THIAZOLE AS AN ISOPRENOID ANALOGUE

*The Louisiana State University and Agricultural and Mechanical Col.* PH.D. 1981

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __✓__.

1. Glossy photographs or pages _____
2. Colored illustrations, paper or print _____
3. Photographs with dark background _____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages __✓__
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print __✓__
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

# ON THE BIOSYNTHESIS OF IRREGULAR TERPENES: MECHANISTIC STUDIES EMPLOYING THE THIAMINE THIAZOLE AS AN ISOPRENOID ANALOGUE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanucal College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biochemistry

by
Steven J. Jungk
B.S., Louisiana State University, 1976
May, 1981

ACKNOWLEDGEMENTS

I would like to thank the members of my committee: Dr. Risinger, for his guidance throughout my research; Drs. Allen, Younathan, and Blakeney for their support and encouragement during my graduate career; and a special debt is acknowledged to Dr. Fischer, whose advice and understanding came at a time when I needed it the most.

My heartfelt thanks go to my family and friends who helped me through the rough times, especially: my parents, who, through their love, time and devotion, made it possible for me to get this far; Mr. R. J. Voll (and Catnip), whose friendship and good cheer made the last five years enjoyable; Dr. K. Karimian, wherever he may be; Mr. M. B. Doughty, whose help was always appreciated, if never expressed; and Ms. Alyce P. Lappin, who, throughout these last months, helped me when she could and whose loving support made the unbearable bearable. Lastly, I would like to thank Ms. Ella Kelley for typing this manuscript on such short notice and for tolerating my illegible scrawl.

*To Alyce,*

*For being there when I needed her.*

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

## ABSTRACT

The irregular terpene, 2,6,9,13-tetramethyl-6-vinyl-tetradeca-2,8,12-trien-7-one (18), and the terpenoid, 1-phenyl-2,6-dimethyl-2-vinyl-hept-5-en-1-one (19) were synthesized by the Continuous Flow Reformatsky method using geranoyl chloride and benzoyl chloride, respectively. Geranyl bromide was used to generate the required organo-zinc adduct. Citral and geranyl bromide produced 3,6,9,13-tetramethyl-9-vinyl-tetradeca-3,7,12-trien-6-ol (17) by this method. Attempts to isomerize 17 to 2,6,9,13-tetramethyl-6-vinyl-tetradeca-2,8,12-trien-7-ol failed. It is postulated that 17 is more stable due to steric compression around the quaternary carbon. Comparison of 18 and the terpene isolated from yeast enzyme preparations by Bell[135] showed them to be identical.

Attempts to synthesize 2-hydroxygeranyl thiamine by the addition of thiamine to citral failed. Cinnamaldehyde also failed to react with thiamine to yield 2-hydroxycinnamyl thiamine. Thiamine and 3-methylbenzothiazolium hemisulfate both react with benzaldehyde to produce 2-benzoyl-3-(2-methyl-4-aminopyrimidin-5-yl) methyl-3a-methylperhydrofuro [2,3-d] thiazole (5) and 3-methylbenzothiazolinyl phenyl ketone (8), respectively. Since 8 is formed via a carbene, a similar proposal is made to account for the behavior of thiamine.

Tritiated citronellal, prepared by the selective hydrogenation of the α,β-olefin of tritiated citral, was reacted with thiamine to produce labelled 2-(1-hydroxy-3,7-dimethyl-1-oct-6-enyl) thiamine chloride hydrochloride ($^{3}$H-1). When $^{3}$H-1 was incubated with geraniol or farnesol 7% of the label was incorporated into 2,6,9,13-tetramethyl-6-vinyl-2,12-tetradecadien-7-one (18a) and 2,6,9,13,17-pentamethyl-9-vinyl-octadeca-2,11,16-trien-8-one (26), respectively. Labelling studies suggest that squalene synthetase is less specific for chain length of the terpene substrate at Site II, than at Site I. Also the thiamine thiazole mimics an isoprenoid moiety of farnesol and the cofactor is not involved in squalene synthesis as proposed by Woodward.[128,129]

The terpenoid, 2-(1-hydroxy-3,7-dimethyloct-6-enyl)-4-methyl-5-(2-hydroxyethyl) thiazole (2) was found to be inactive in yeast enzyme preparations.

The formation of 2-alkyl derivatives of thiamine via the Hantzsch Thiazole Synthesis was investigated. Using model systems, S-phenacyl thiobenzanilide and S-(*p*-chlorophenacyl) thiononanilide were synthesized but failed to yield the desired thiazolium salts. Steric hindrance of the substituents was responsible for this failure.

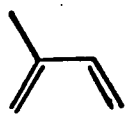
Thus, based on the isolation of 18 from the cell-free yeast enzyme preparation, it is suggested that while thiamine is not involved in tail-tail dimerization, the vitamin may play a role in the formation of artemisia ketone and bakuchiol.

# I. INTRODUCTION

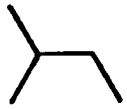
In almost every form of life, terpenes have been found. These compounds, although markedly varied in physical properties, bear similarities which distinguish them from other natural products. Terpenes possess the repeating five carbon unit of isoprene (Fig. 1). As a result, the names: hemi-, mono-, sesqui-, di-, tri-, and tetraterpene have been associated with five, ten, fifteen, twenty, thirty and forty carbon compounds, respectively.

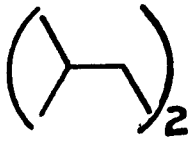
Man's interest in terpenes is probably as old as man himself. Since the dawn of written history, records have shown man's interest in essential oils. The essence of turpentine was known to the ancient Egyptians. Folk medicine frequently used bitter herbs, the constituents of which contain sesqui- and diterpenoids. Manuscripts, dated in the eleventh century, document the use of camphor in Europe. Various treatises from the fourteenth to the seventeenth century mention the aromatic properties of a variety of essential oils.[1]

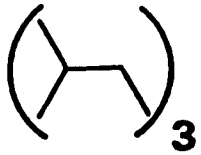
With the advent of modern chemistry, terpenes were subjected to stricter analyses. By the relatively primitive techniques available, chemical pioneers showed the empirical formulae of terpenes to be in multiple of $C_5H_8$.[1-3] In 1884, Tilden[4] showed that isoprene was formed by thermal decomposition of a variety of

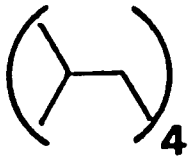


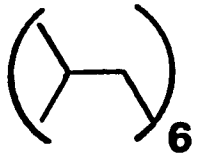
isoprene

hemiterpene 

monoterpene 

sesquiterpene 

diterpene 

triterpene 

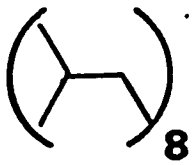
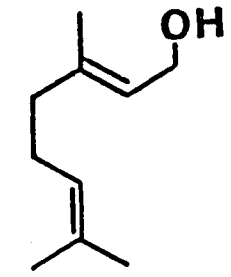
tetraterpene 

Figure 1. Structural relationship among terpenes.

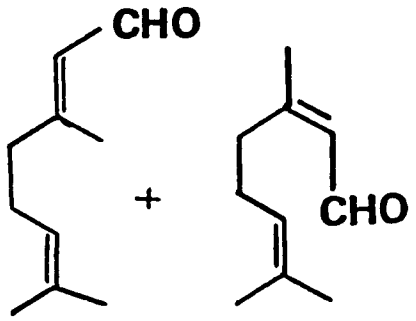
monoterpenes. As new techniques in chemistry were developed, terpenes were subjected to degradative analyses to determine their structures[5-12] and many simple isoprenoids were characterized (Fig. 2).

With a large variety of cyclic and alicyclic terpenes having been characterized, Wallach[13] suspected that all terpenes occur in multiples of isoprene, assigning a head and a tail to this unit (Fig. 3). Several years later, Ruzicka proposed the isoprene rule,[14-16] which states that all terpenes are linked together in a regular fashion by the attachment of the head of one unit to the tail of another, forming an acyclic compound. Thus dextropimaric acid (Fig. 4), a tricyclic diterpene, can be formed from geranylgeraniol, an alicyclic diterpene.

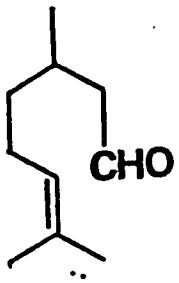
Although Ruzicka modified his original idea to the "biogenetic isoprene rule"[17] to account for irregularities found in many terpene skeletons, these early concepts could not explain the $C_{30}H_{50}$ metabolite isolated by Tsujimoto[18] from shark livers. This compound, called squalene, was shown to be an acyclic triterpene by Heilbron[19] and was chemically synthesized by Karrer[20] by reductive dimerization of farnesyl bromide, clearly showing the irregular (tail-tail) bonding between the two isoprenoid groups. Biosynthetic studies of terpenes were initiated when squalene was implicated in cholesterol production.[19,21] In one of the first studies using isotopic labelling, Sonderhoff[22] fed trideuteroacetic acid to yeast and found sterols with a large amount of deuterium incorporated.



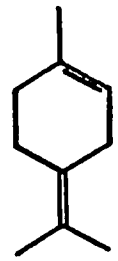

geraniol



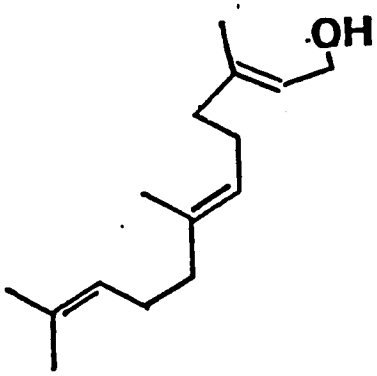

citral



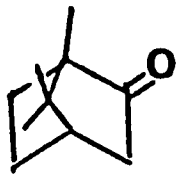

citronellal



limonene




farnesol




camphor

Figure 2. Simple terpenes.

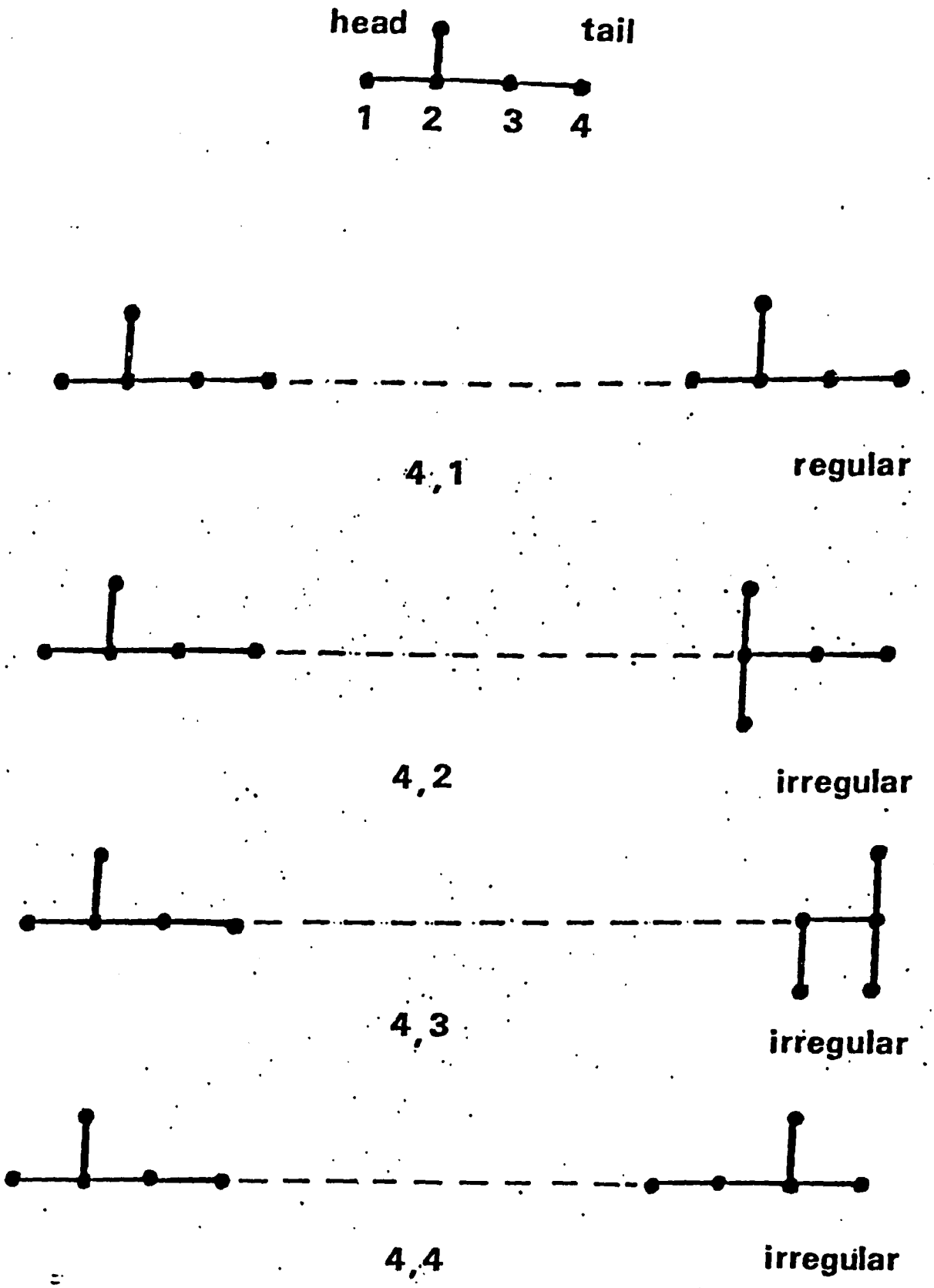



Figure 3. Various linkages of isoprenoid units.

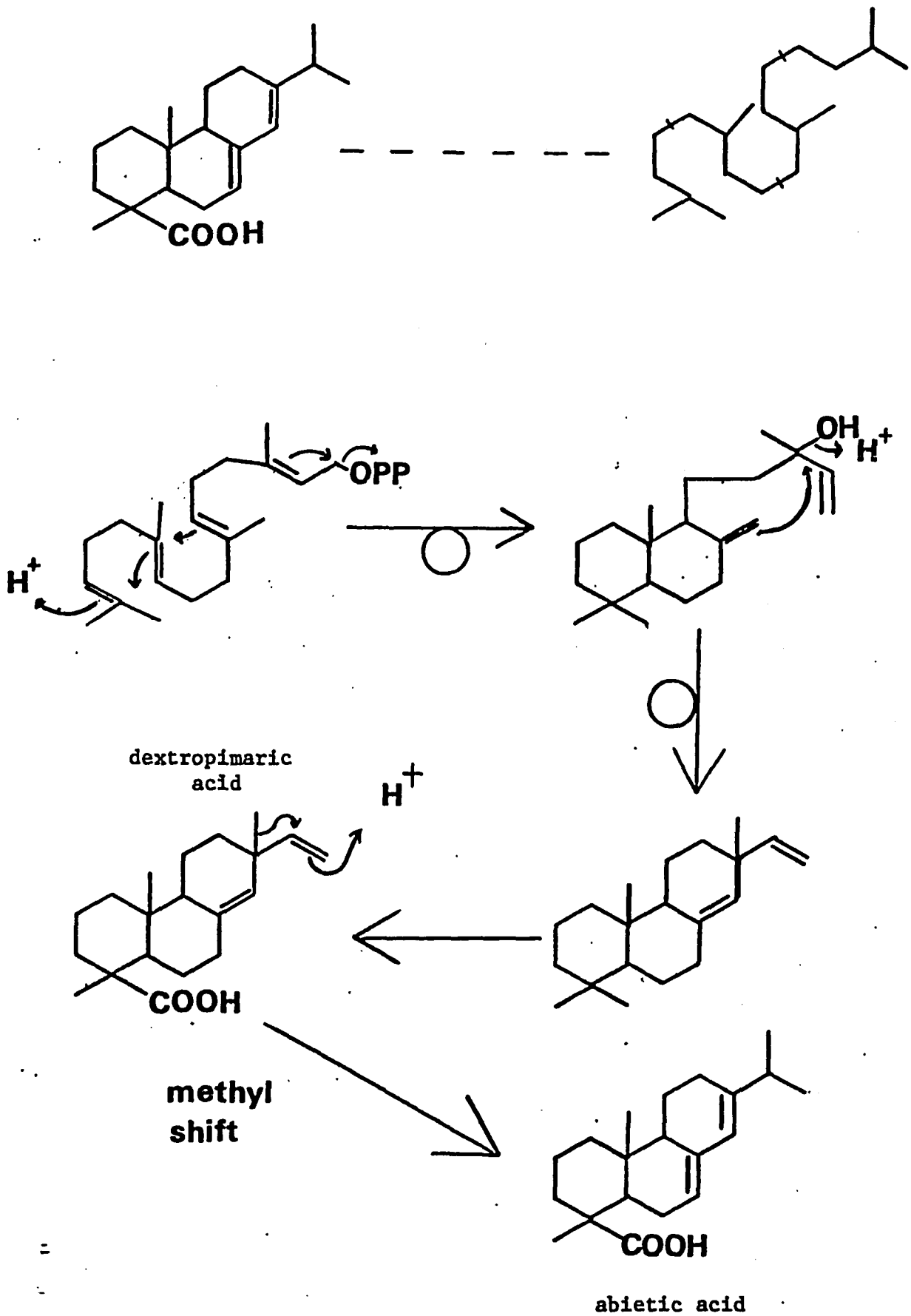



Figure 4. Biosynthesis of dextropimaric and abietic acids.

For 15 years, very little work concerning simple terpene biogenesis was done. Then in 1952, Bloch,[23,24] and Cornforth[25] showed that labelled acetate could be incorporated into squalene (Fig. 5). Bloch[26,27] subsequently demonstrated that squalene was the biogenic precursor to cholesterol.

At this point there was a veritable explosion of research in terpene biosynthesis. Initial clues into the biochemical equivalent of isoprene or "active isoprene" were found when a compound, called mevalonic acid (MVA), characterized by Tavormina[28,29] in 1957, could be substituted for acetate in *Lactobacillus acidophilus*.[30-33] Due to the structural simplicity of mevalonic acid, it was not long before its biogenesis from acetyl CoA was elucidated.

The biosynthesis of MVA[34] (Fig. 6) from acetyl CoA proceeds via a Claisen condensation of two acetate groups, followed by an aldol condensation with a third. The resulting compound, β-hydroxy-β-methylglutaryl CoA, is subsequently reduced by the allosteric enzyme HMGCoA reductase to give mevalonic acid. The ubiquituous nature of this compound is further evidence of its key role in terpenoid and steroid production. Bloch[35] found that MVA could be converted to isopentenyl pyrophosphate (IPP). Two distinct steps are thought to occur in this conversion.[1] First, the C-5 hydroxyl group of mevalonic acid is pyrophosphorylated sequentially:[36] one ATP is consumed to produce the monophosphate, followed by the subsequent pyrophosphorylation with a second ATP (Fig. 7). The second step is the monophosphorylation of the C-3 alcohol with a

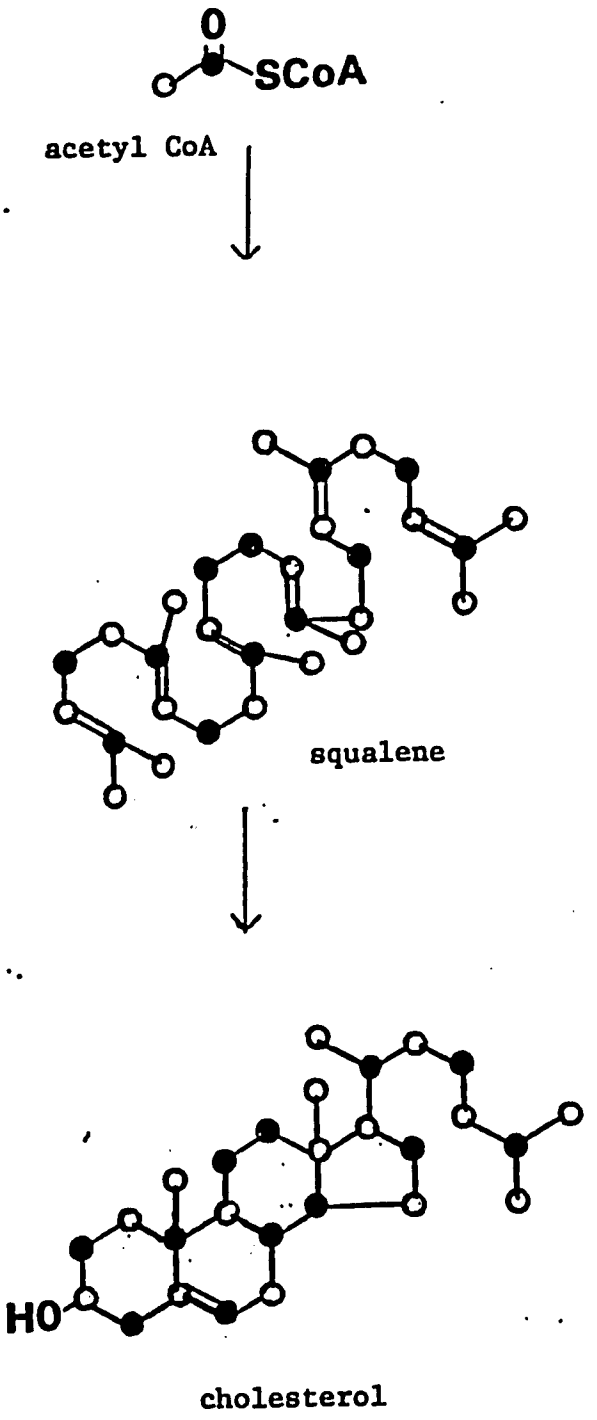



Figure 5. Labelling pattern in cholesterol.

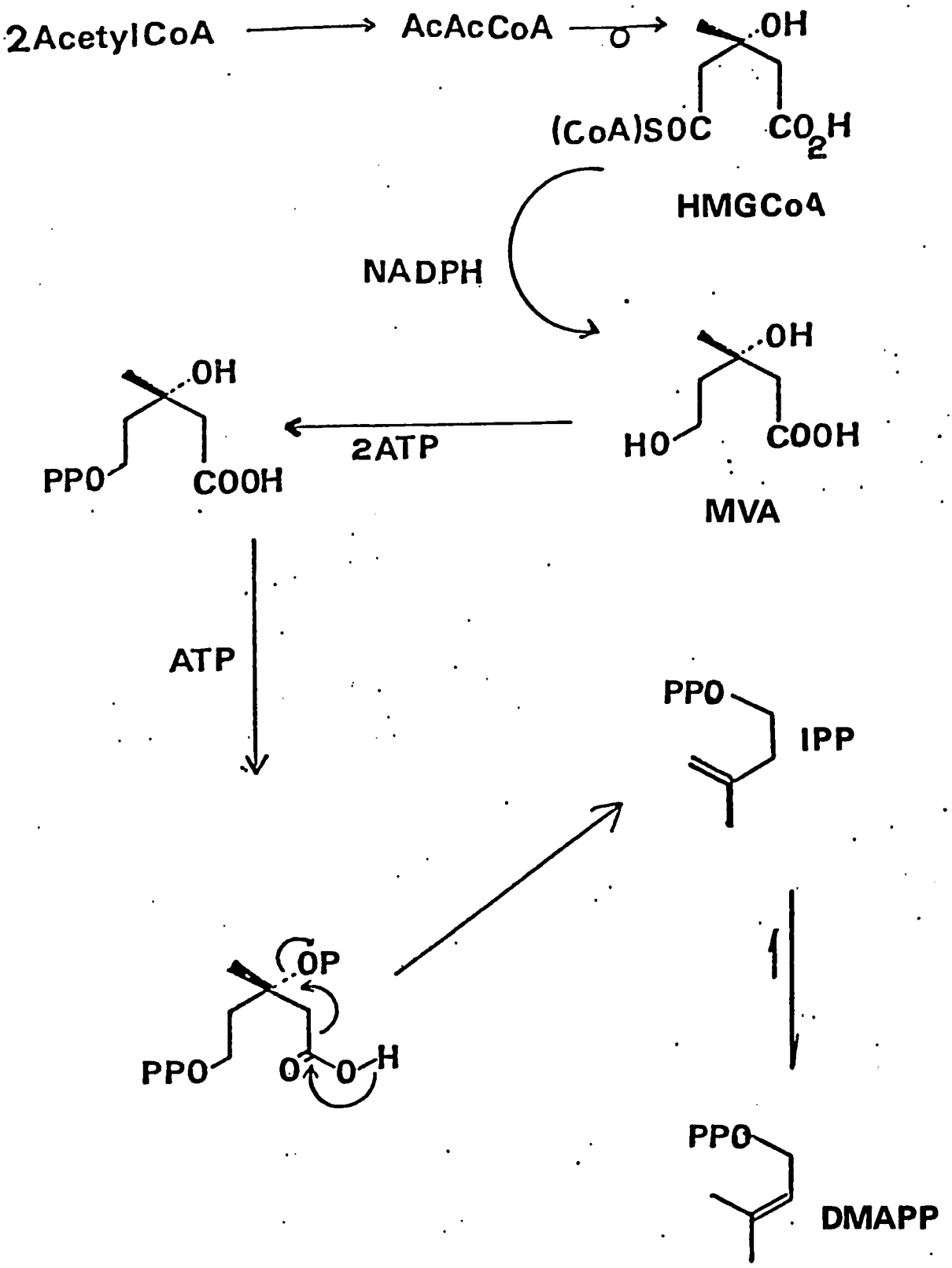



Figure 6. Biosynthesis of mevalonic acid and the hemiterpene monomers.

third ATP, followed by concerted decarboxylation-dephosphorylation, leaving IPP.[37,38]

Isopentenyl pyrophosphate exists in equilibrium with its isomer, dimethyl allylpyrophosphate (DMAPP). The isomerization requires no cofactors and is catalyzed by isopentenol isomerase.[39] Using DMAPP as a starting template, attack of the olefin of IPP on the C-1 pyrophosphate of DMAPP occurs, generating a carbonium ion which is then satisifed by proton removal to give geraniol (Fig. 7). The overall reaction is viewed as an $S_N2$-$E_1$ condensation.[40] By addition of successive isoprenoids, farnesol, geranylgeraniol, and higher terpenes can be synthesized.

The preceeding steps outline the mechanism which is unequivocally considered the normal mode of regular terpene biosynthesis. The points which follow, however, have been highly controversial. Debates still rage concerning these facets of terpene biosynthesis which are commonly considered as "irregular" terpene biogenesis.

There exists in nature a large body of terpenes which do not possess the normal head-tail linkage found in the so-called "regular" terpenes. Many of the abnormal or "irregular" terpenes have been shown to arise from regular precursors (see Fig. 3). For example abietic acid (Fig. 4) appears to have a tail-tail (4-4) linkage in its carbon backbone. Biosynthetic studies by Wenkert[41,42] have shown that this irregular linkage does not arise

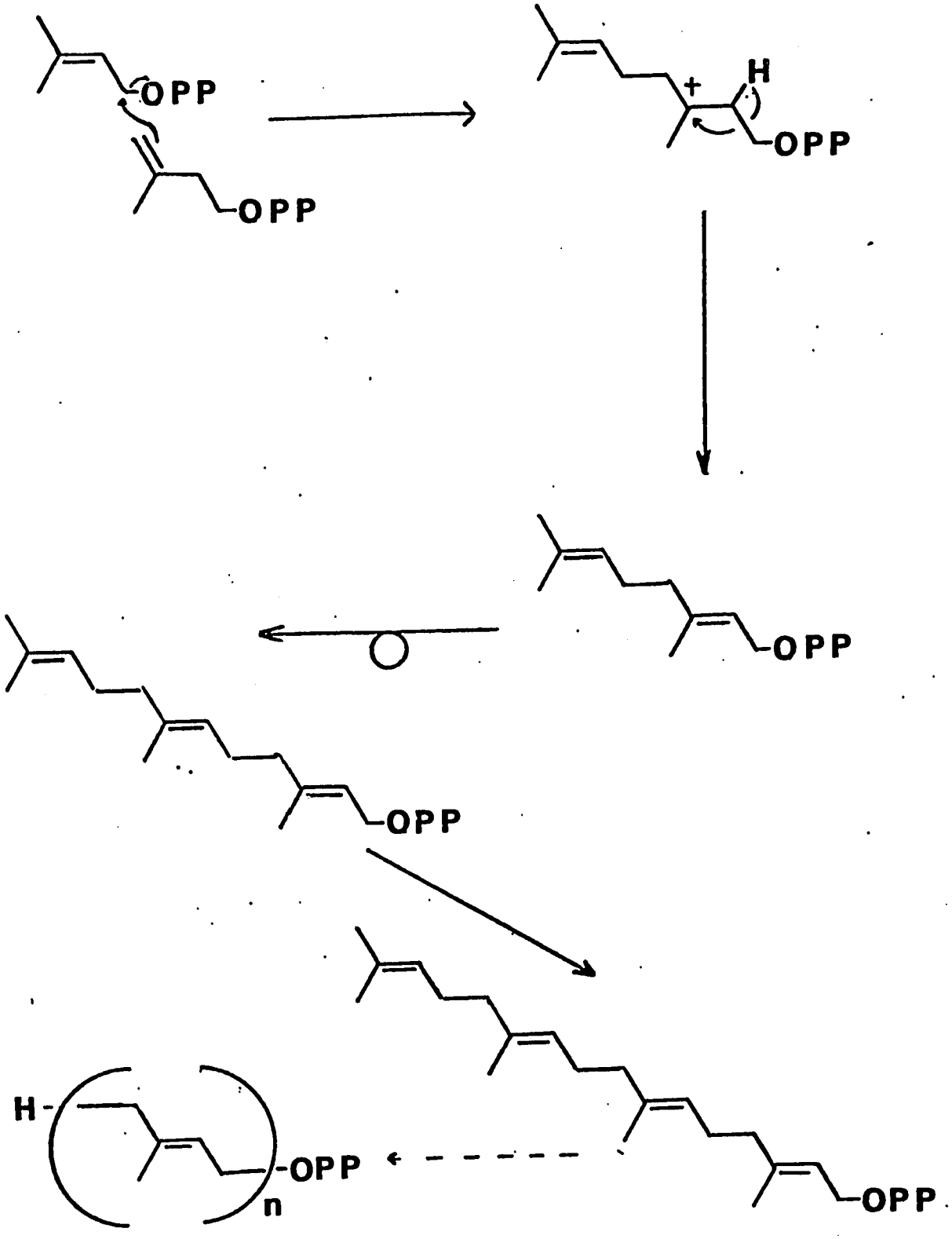



Figure 7. Biosynthesis of regular terpenes.

from an unknown pathway bonding the C-1 carbon of farnesyl pyrophosphate with that of DMAPP. Rather, it is formed from the sigmatropic methyl shift to alleviate the charge of a secondary carbocation. Abietic acid, at least in this respect, cannot be considered irregular as it arises from dextropimaric acid.

The same biogenetic approach can be applied to secologanin.[43,44] Although the heavily oxidized skeleton and the hemiacetal glycoside make casual inspection difficult, a close look at the structure of this molecule reveals ten carbons bonded in a non-isoprenoid fashion. After considerable effort, it was found that this compound arises from the iridoid class of monoterpenes which are derived from citronellal (Fig. 8). The many examples of alkyl group rearrangements do not, however, shed any light on the biosynthesis of squalene and phytoene from farnesyl pyrophosphate and geranylgeranyl pyrophosphate, respectively. Details concerning the mode of the tail-tail dimerization required was not forthcoming until the importance of squalene was realized.

Quite a few speculations about its role in cholesterol biosynthesis existed for many years,[19,21,45,46] but it was not until 1953 that the first real evidence came to light implicating squalene in steroid biosynthesis. After numerous failures Bloch[26] showed that this triterpene could be synthesized by rat livers and this labelled material was shown to be converted to cholesterol.[27]

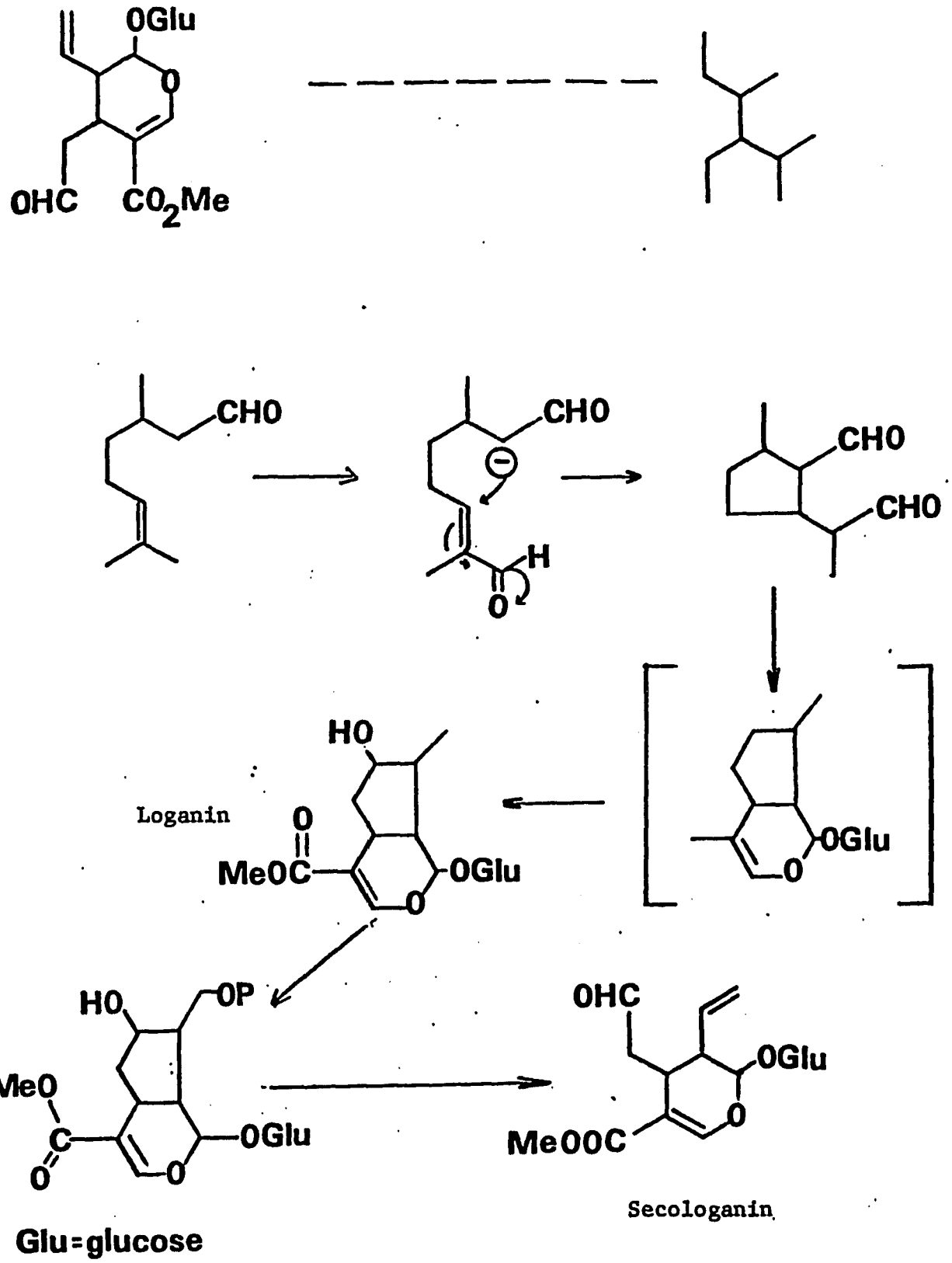



Figure 8. Biosynthesis of loganin and secologanin.

Once this fact was established, research into mode of reductive dimerization of farnesyl pyrophosphate was seriously undertaken. Lynen[47] found that NADPH is a required cofactor. Rilling[48] discovered a C-30 compound accumulates in the absence of NADPH. The initial structure assigned was later shown to be incorrect[49] and was modified to what is now known as presqualene alcohol[50,51] (Fig. 9). These same researchers[52] chemically synthesized this compound and found it to be active as a steroid precursor in yeast preparations. The discovery of presqualene came after extensive work by Cornforth and Popják.[53-57] Therefore, all mechanisms[51,52,58-60] concerning the formation of squalene via presqualene must include these considerations. Van Tamelen's mechanism[60] is representative of these proposals (Fig. 9). Subsequent conversions of squalene to cholesterol first involves cyclization similar to dextropimaric acid (Fig. 4), and methyl-hydride shifts to give lanosterol (Fig. 10) which are identical steps in form, to those shown for abietic acid (Fig. 4). Demethylation, isomerization of the endocyclic olefin to the Δ5 position and reduction of the acyclic double bond generates cholesterol (Fig. 10).

Upon the discovery of presqualene, the search for an equivalent compound in carotene biosynthesis was initiated. Since the tail-tail linkage in phytoene is similar to that found in squalene, it was assumed that their biosynthetic pathways were similar.

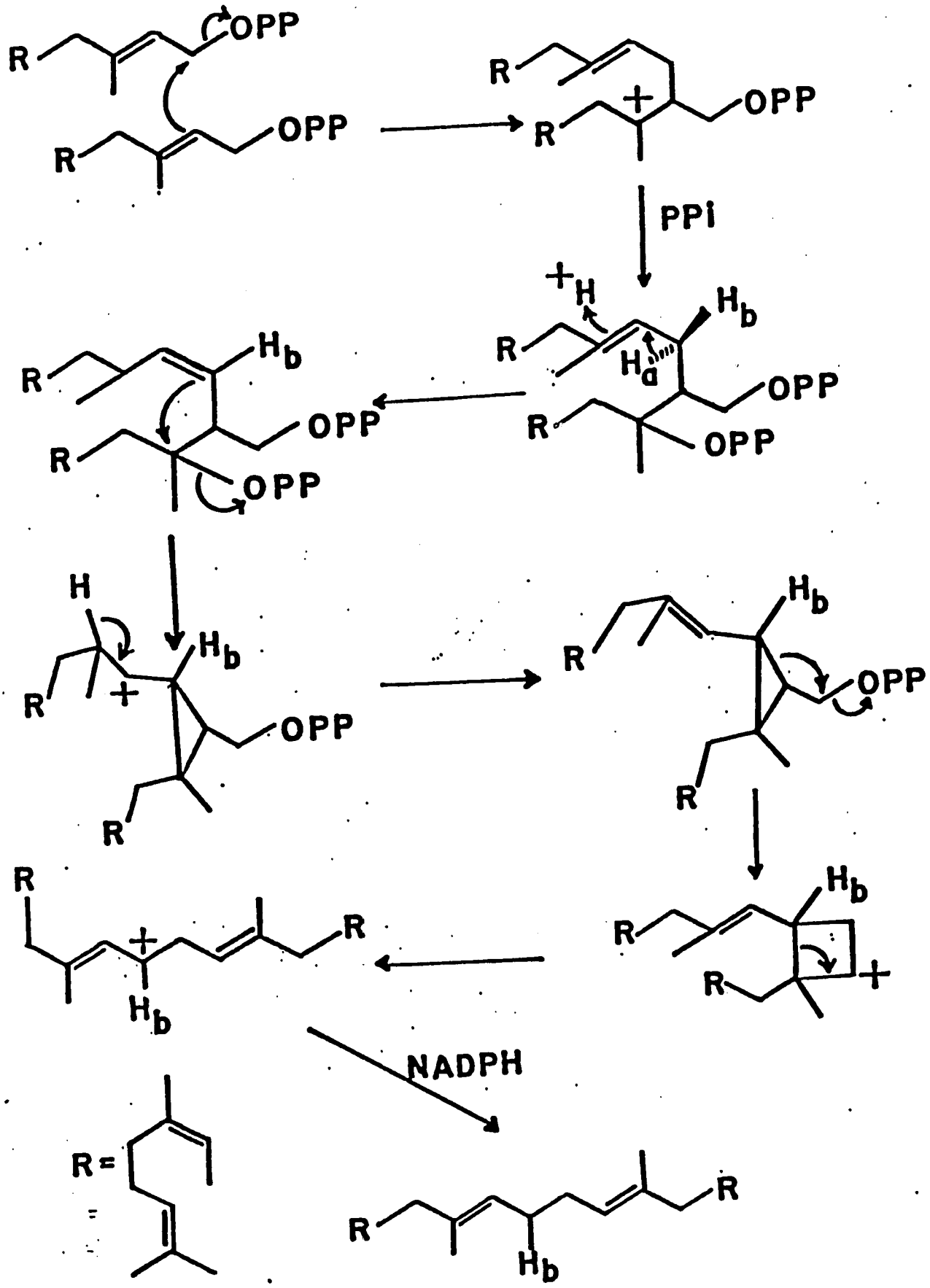



Figure 9. Van Tamelen's mechanism for the biosynthesis of squalene.

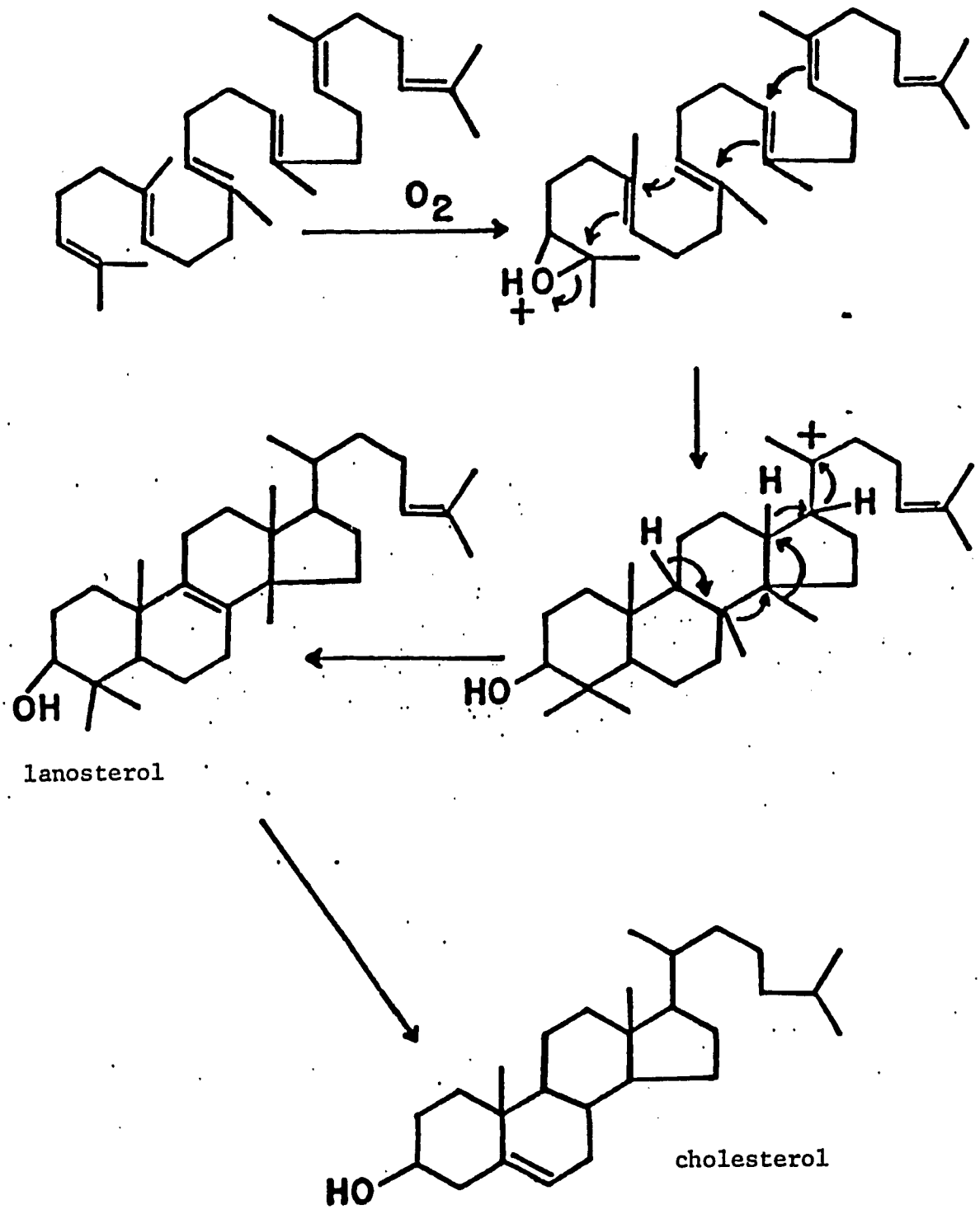



Figure 10. Biosynthesis of cholesterol from squalene.

Upon the discovery of prephytoene, this assumption was borne out[61,62] with the major difference being the loss of the 1-pro-S hydrogen to form the olefin (in phytoene) rather than reduction with NADPH (in squalene). Formation of this molecule leads to the 15-15' cis isomer (Fig. 11), but in some microbial sources the all trans isomer, formed from the loss of the pro-R hydrogen, has been isolated.[63] Conversion of phytoene to lycopene occurs through dehydrogenation.[61,62] The final steps, leading to the carotenes is the cyclization of the two terminal geranyl groups[64] (Fig. 11).

Presqualene and prephytoene are not the only terpenoid compounds which possess the unusual cyclopropyl ring structure (Fig. 12). This compound, chrysanthemyl alcohol, is composed of the two C-5 units. Although no C-10 tail-tail terpenes have been isolated, it is speculated that this compound gives rise to four categories of irregular terpenes[65-67] (Fig. 13). These are classified as chrysanthemyl,[68-70] artemisyl,[68,71-75] lavandulyl,[68] and santolinyl[68,76-79] compounds. Of the many theories developed to account for the origin of these compounds,[65,81-84] the most attractive of these proposals is put forth by Bates.[65] It has received support from other groups[66,67] and chemical models substantiate his views.[85,86] In this biochemical system Bates postulates that two equivalent DMAPP groups condense in a manner similar to the condensation of two farnesyl pyrophosphate molecules in the formation of presqualene. The resulting cyclopropyl intermediate (chrysanthemyl pyrophosphate) can give rise to the four different categories of

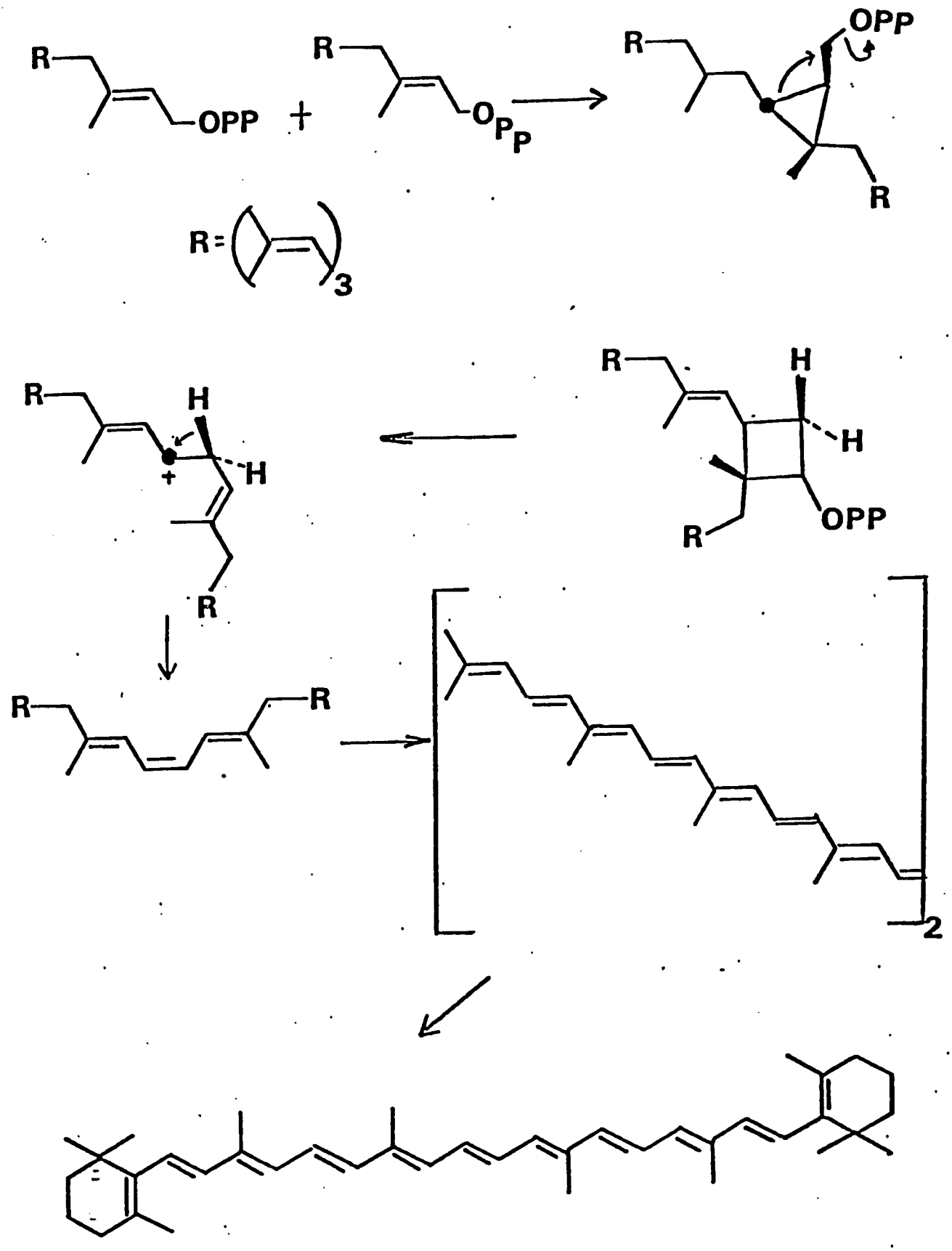
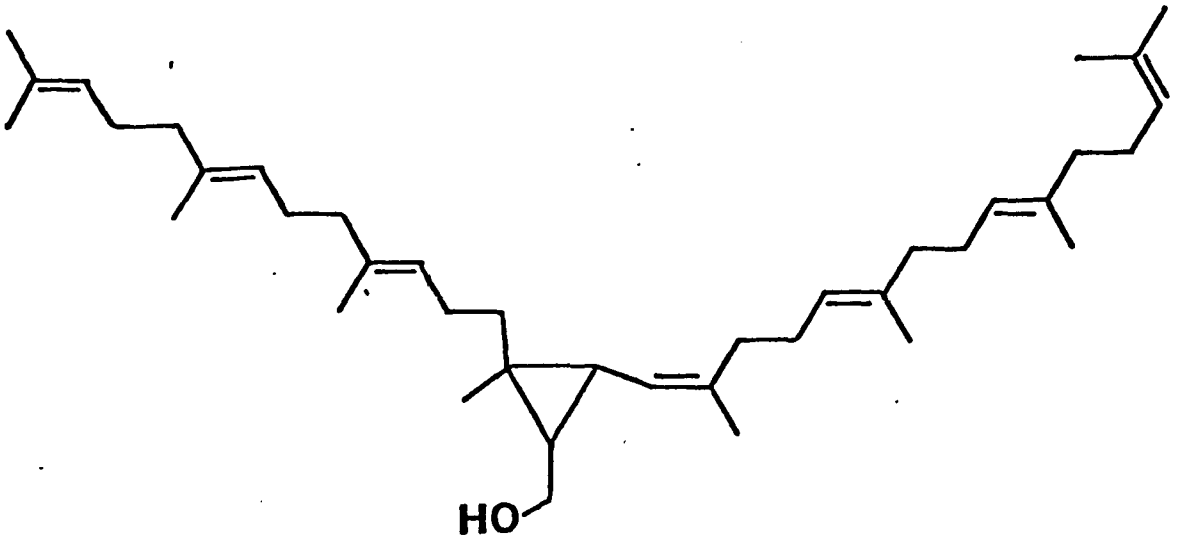
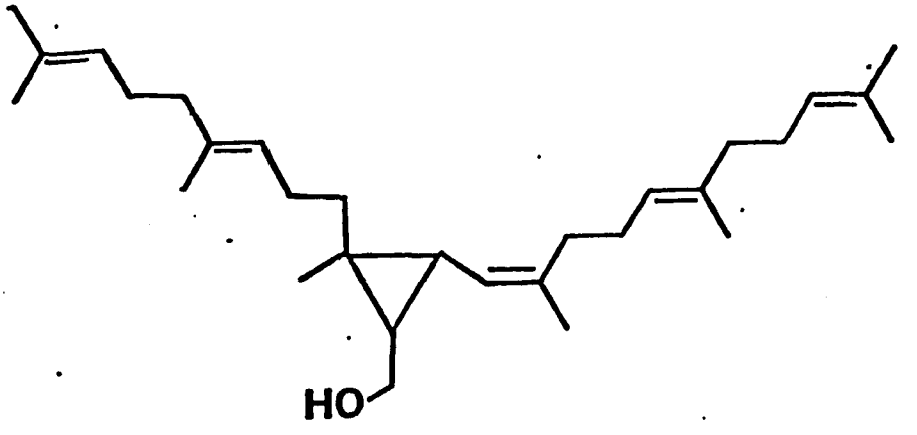


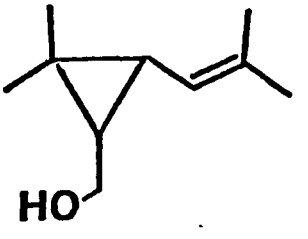

Figure 11. Biosynthesis of carotene.




prephytoene alcohol



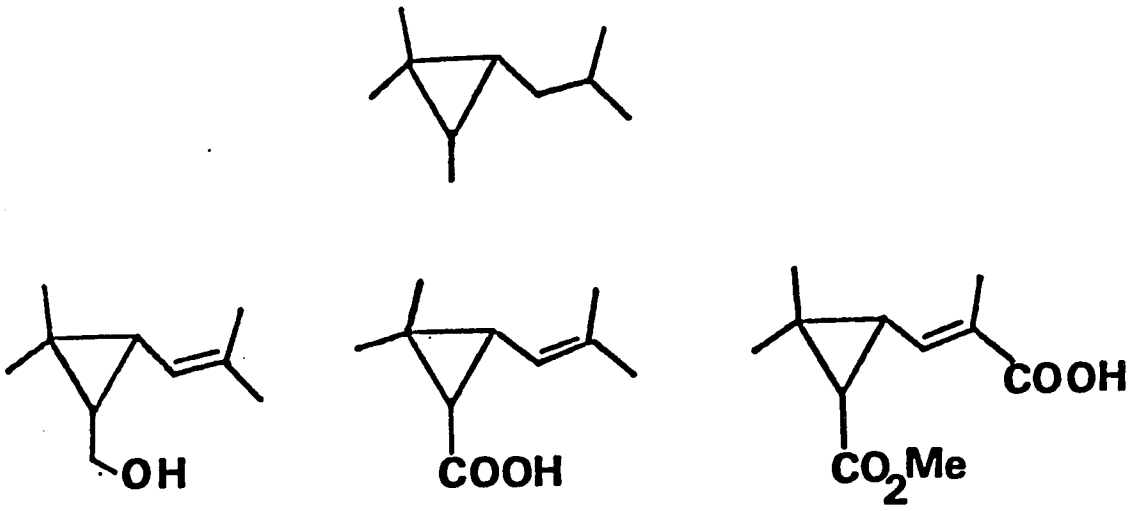

presqualene alcohol




chrysanthemyl alcohol

Figure 12. Comparison of natural cyclopropyl methanols.

Chrysanthemyl




Artemisyl

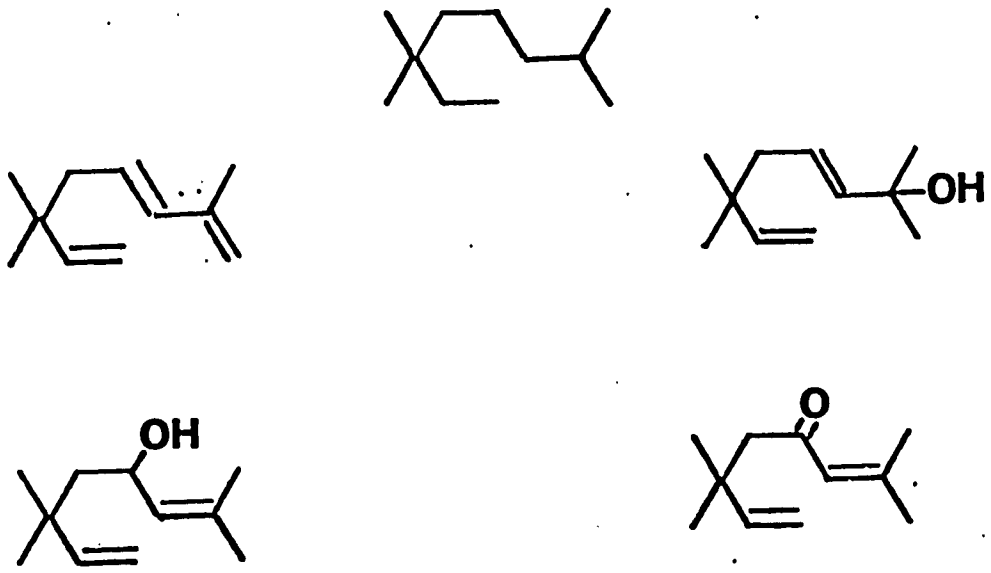
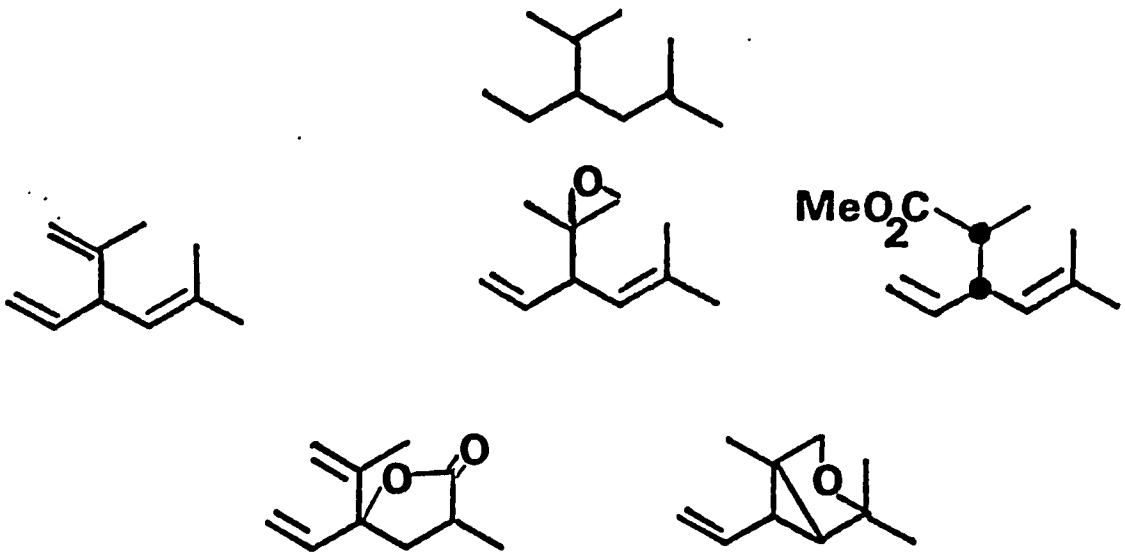



Figure 13. Irregular monoterpenes.

Santolinyl




Lavanulyl

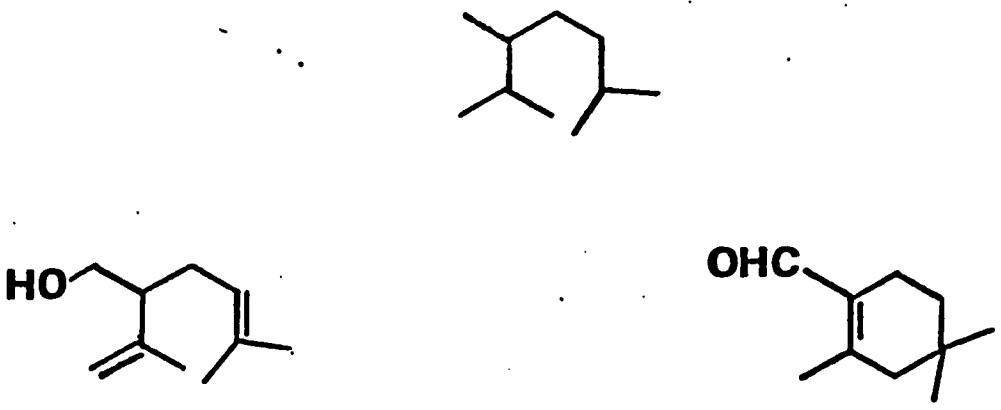
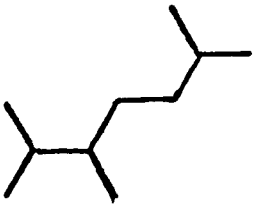



Figure 13 (continued)

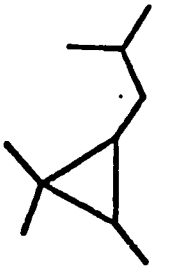
irregular terpenes described above[54,69,85,88] (Fig. 14). Chrysanthemic acid has been shown to be a metabolite of the corresponding alcohol.[68] Furthermore, studies using cell-free enzyme preparations from Artemisia and Santolina species have shown the interconversion of artemisia alcohol (and ketone) and chrysanthemyl pyrophosphate.[87] The formation of this irregular terpene skeleton is thought to arise by ring opening of the cyclopropyl carbonium ion. The santolina skeleton can arise by ring opening, but with cleavage of another bond. Lavanulyl skeletons arise in a different fashion. (See Fig. 14.)

A modified version of this theory is favored by Epstein and Poulter[68] (Fig. 15) in which attack of one DMAPP group upon another generates a tertiary cation, which is alleviated by an electron donating group, "X". Elimination of "X" may proceed in two ways: by direct removal to generate the lavanulyl system or 1,3 elimination to generate the chrysanthemyl skeleton. From this intermediate, artemisyl and santolinyl skeletons may arise.

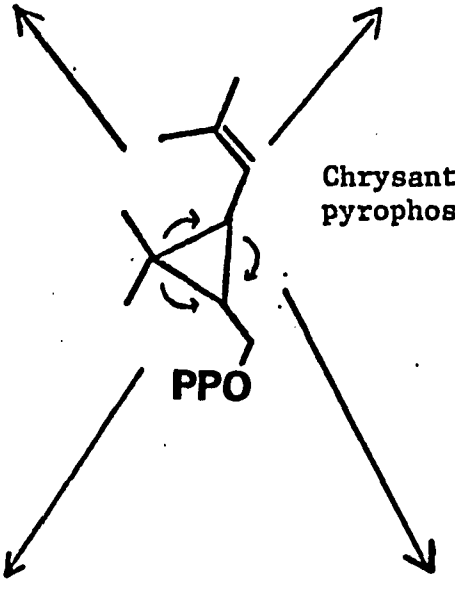
Several observations are not consistent with either theory. First, the interconversion of the four classes of irregular terpenes has not been observed *in vivo*.[54] Second, many labelling studies have shown asymmetric incorporation into these irregular terpenoids.[66,67,86-92] Banthorpe and his coworkers have contributed much to this field; they have found when MVA,[66] IPP, DMAPP,[88,89] and dimethylvinylcarbinol (DMVC)[88] are used as precursors of artemisia ketone, the tracer is predominantly found



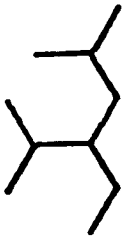
Lavanulyl



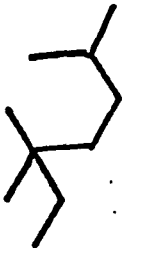
Chrysanthemyl




Chrysanthemyl
pyrophosphate



Santolinyl



Artemisyl

Figure 14. Bates' mechanism for irregular terpene biosynthesis.

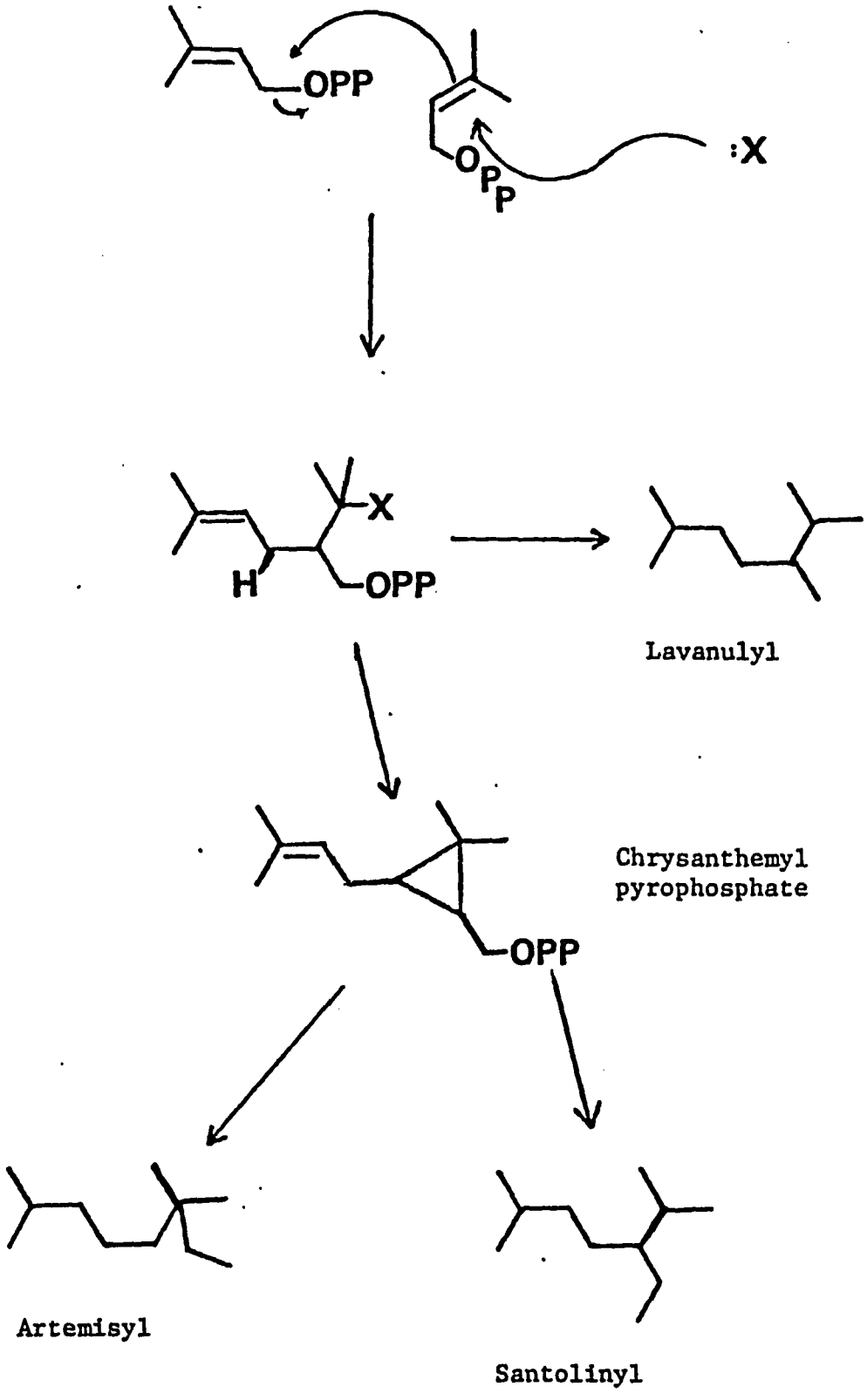



Figure 15. Epstein and Poulters' mechanism for the biosynthesis of irregular terpenes.

in the non-ketone isoprenoid moiety (Fig. 16). Label in the ketone portion varies from 0% to 20%, depending on the substrate and species of plant used (*S. chamaecyparrisus* or *A. annua*). To explain these anomalous results, the theory of compartmentalization was developed[68,93,94] (Fig. 17). This theory has found acceptance, not only to explain the labelling pattern of irregular terpenes, but many regular terpenes as well.[95-105] The hypothesis of separate pools of isoprenoids was tested by several groups,[48,106-109] with the most dramatic results obtained by Wuu and Baisted.[106] They found that immediately after feeding, geraniol is almost equally labelled in both $C_5$ units, although very little new geraniol was synthesized, but after 12 hours, the level of labelled geraniol was much higher and 78% of the label was found in the IPP portion of geraniol. After 24 hours, 75% of the geraniol had been turned over and only 59% of the label was found in the IPP portions (Fig. 18). Thus, these investigators postulate that exogeneous MVA may serve as a precursor for IPP, but not DMAPP, in monoterpene biosynthesis.

Non-mevalonate derived isoprenoids may serve as effective substrates in terpenoid biosynthesis and could possibly explain the anomalous labelling pattern found in some terpenes. A number of examples of amino acid participation in isoprenoid biosynthesis have been documented. The isoprenoids angelic and tiglic acid, have been shown to arise from degradation of isoleucine.[109-112] Valine has been shown to be involved in the formation of echimidic

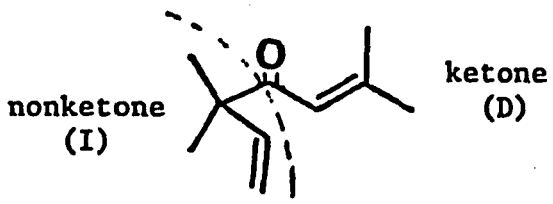



Table I[66,88-90]

| Plant | Substrate | %I | %D |
|---|---|---|---|
| *A. annua* | MVA | 80 | 13 |
| " | MVA | 92 | 10 |
| " | IPP | 96 | 5 |
| " | DMAPP | 89 | 10 |
| " | nerol, geraniol | 10 | -- |
| *S. chamaecyparrisus* | IPP | 93 | -- |
| " | DMAPP | 93 | -- |
| " | DMVC | 80 | -- |

Figure 16. Labelling pattern from various substrates in artemisia ketone.

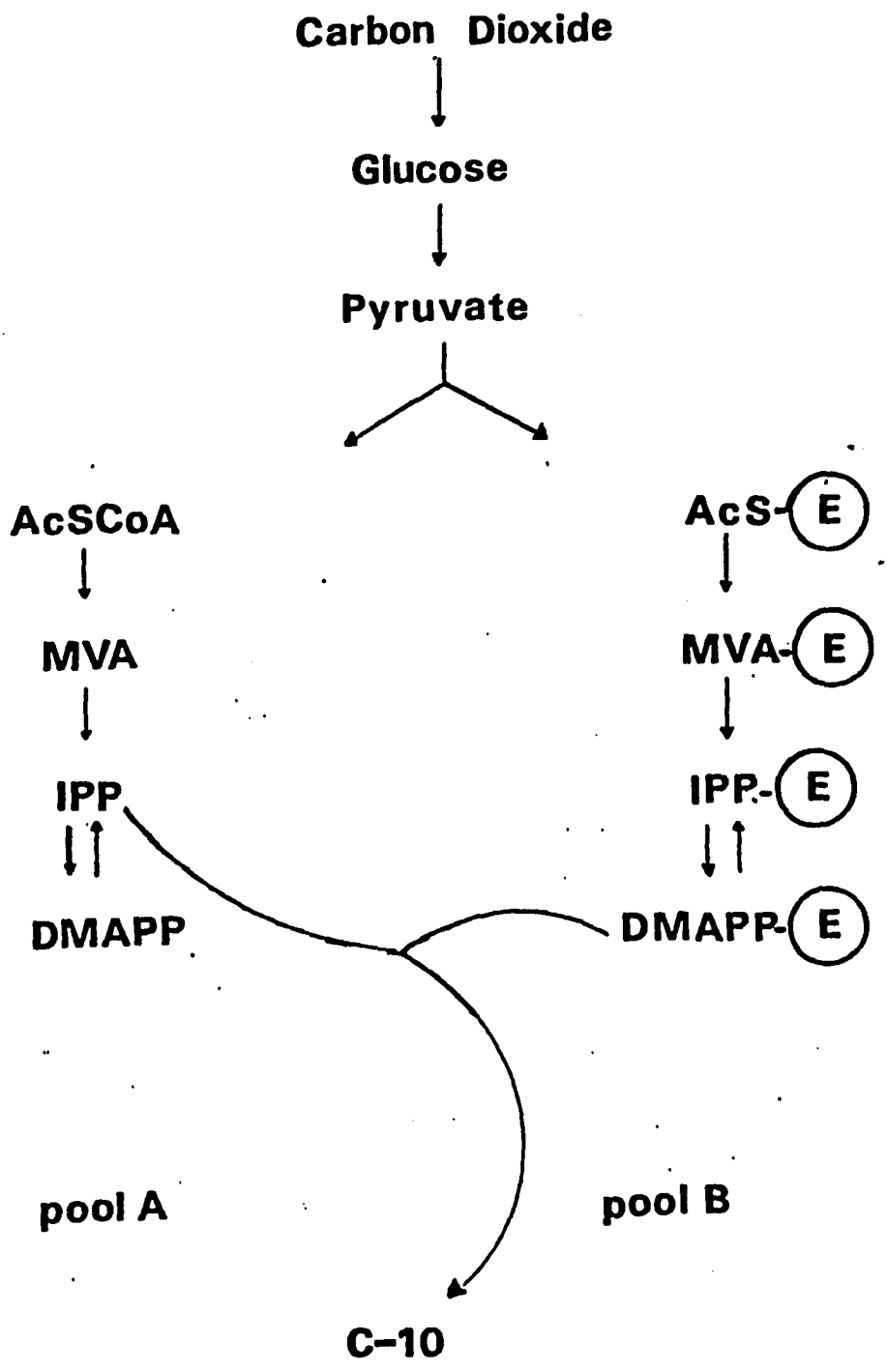



Figure 17. Metabolic pools theorized to account for asymmetric labelling.

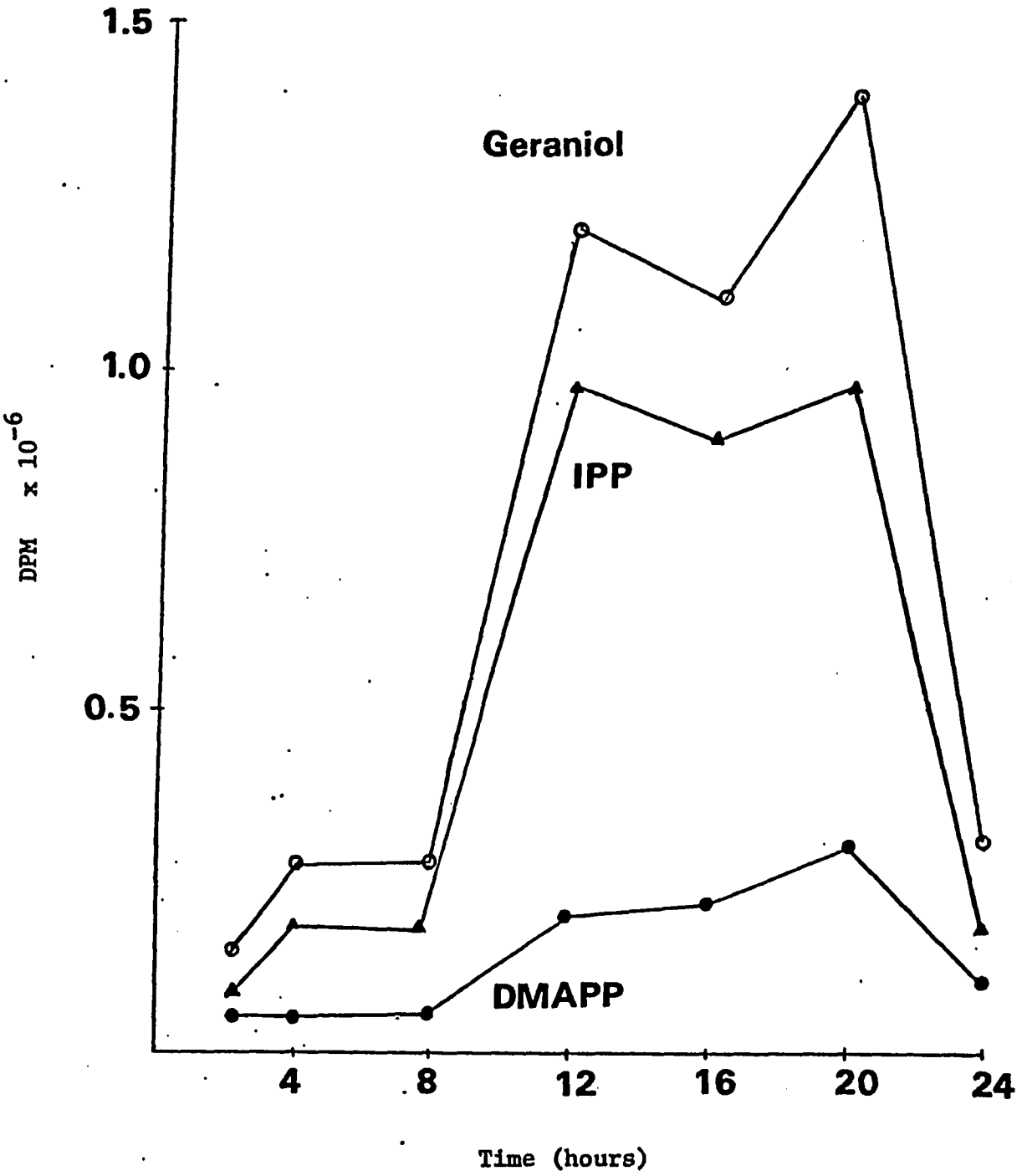



Figure 18. Time course study using $^{14}CO_2$ (2 hour exposure) followed by 22 hour metabolism in ambient air (after Wuu and Baisted).[106]

acid,[113] a compound with isoprene characteristics (Fig. 19) and this amino acid has also been implicated in playing a minor role in steroid biosynthesis.[114] By far the most dramatic evidence comes from labelling studies done with leucine (Fig. 20). Senecioic acid (dimethylacrylic acid), arising from valine and leucine, has been suggested by Suga[95-97] as an intermediate in some IPP production, but this claim has not been independently verified, and as such, has been given little credibility.[94] Hydroxymethyl glutaryl CoA (HMGCoA), produced from senecioic acid by carboxylation and hydration, has been studied in great detail.[115-118] It has been shown that HMGCoA undergoes a retroaldol, resulting in the formation of acetoacetic acid and acetyl CoA, whereupon the acetate formed is lost in the "acetate pool." The results of Yokoyama[119] and Goodwin[120-123] bear this out. It was found that valine could serve as a moderate activator of carotene biosynthesis in the mold *P. blakesleenus*, but leucine was, by far, a more potent activator. Furthermore, it was found that this compound only serves as an activator in the presence of $CO_2$. Carbon dioxide, conversely, is fixed in carotenoid only in the presence of leucine. The pattern of incorporation was extensively studied at each carbon of leucine,[119] showing high incorporation at C-4. Scrambling at the C-2 label is observed as the amount incorporated is less that that at C-4. Unfortunately, experiments using non-mevalonate isoprenoids as substrates for irregular monoterpene biosyntheses are not plentiful. Simpson[124] has found that 3-methylbutanal and

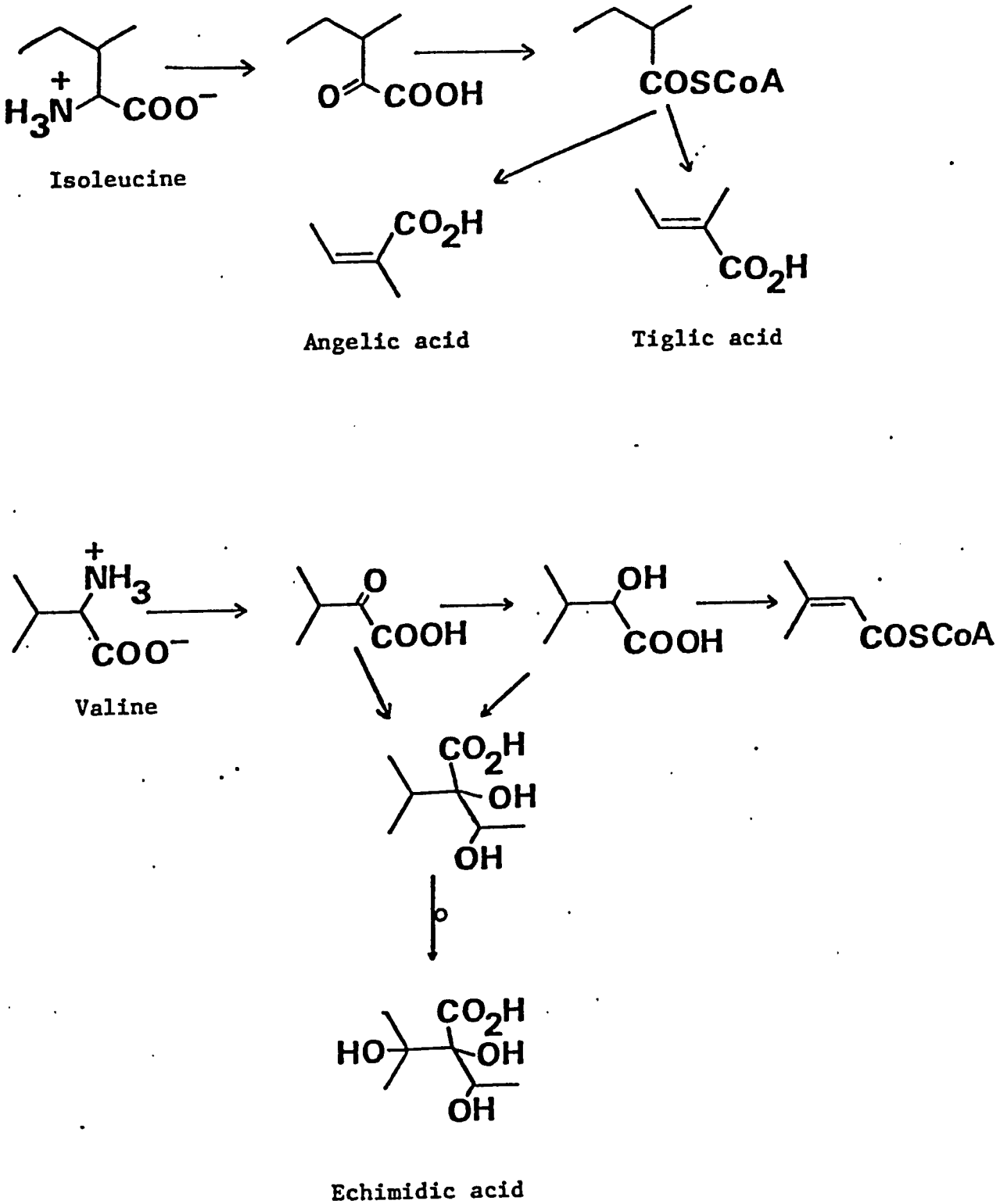



Figure 19. Isoprenoid metabolism from isoleucine and valine

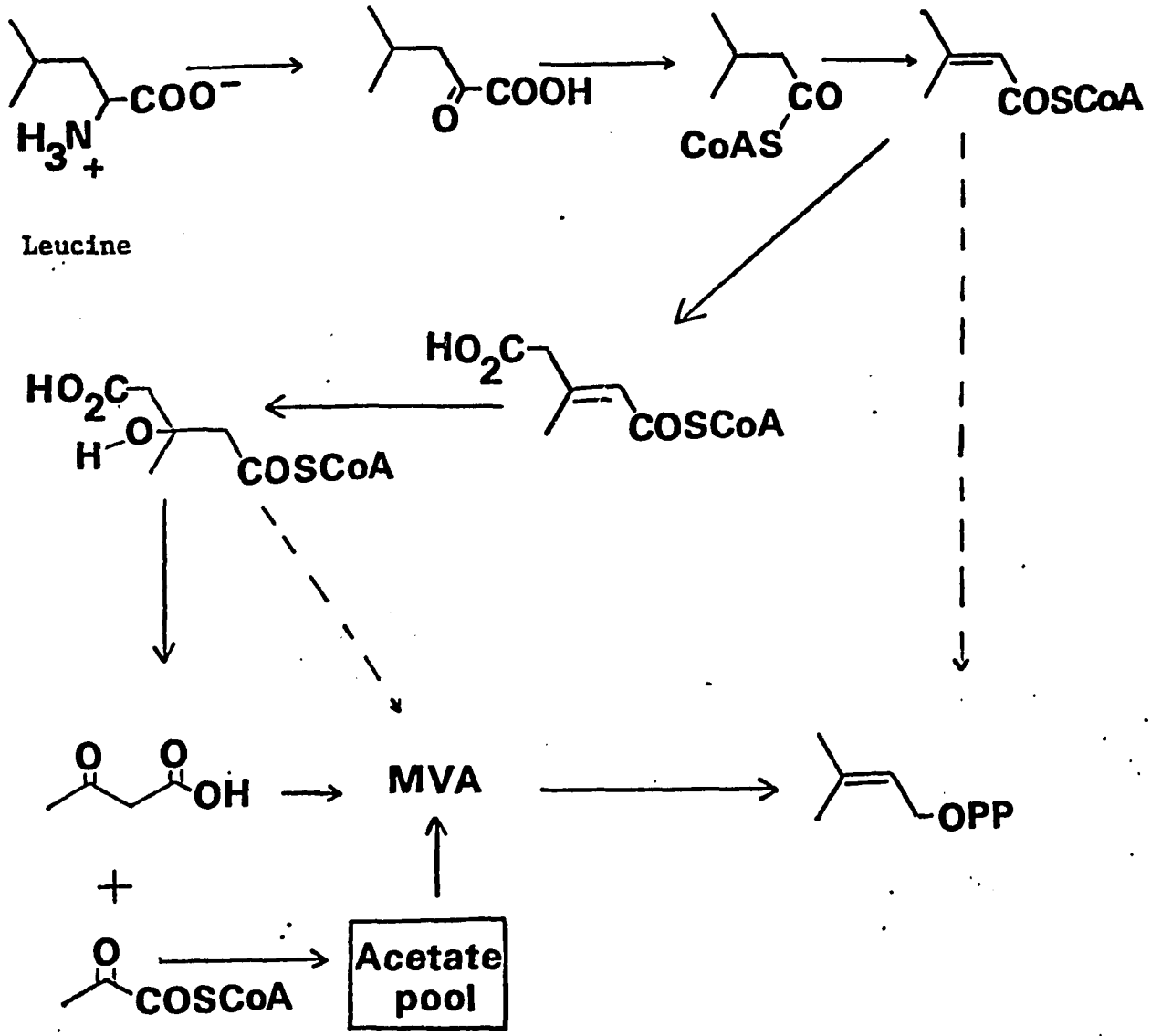



Figure 20. Isoprenoids from leucine metabolism.

hydroxyisocaproic acid are incorporated into artemisia ketone (*A. annua*), but again the label is found to predominate in the non-ketone isoprene unit (Fig. 21).

The discovery of a compound with mixed mevalonate-shikimate origin has led to the development of another theory.[125,126] Bakuchiol (Fig. 22), isolated from the seed pods of *Psoralea corylirolia*, is a terpenoid containing eight carbons thought to arise from tyrosine.[127] This shikimate moiety is linked to the geranyl unit in a morphologically similar fashion as one C-5 unit is bound to the other in the artemisyl family. In order to reduce the number of carbons from nine (as in tyrosine) to eight, decarboxylation of the corresponding α-keto acid, p-hydroxyphenyl-pyruvic acid, must occur. This reaction is mediated by vitamin $B_1$. It is upon this intermediate (Fig. 22) that formation of the artemisyl (or bakuchiol) skeleton is based. $S_N2'$ attack of the generated anion on geranyl pyrophosphate, followed by departure of the thiamine moiety leads to the formation of a phenolic analogue of artemisia ketone. Reduction and elimination would result in the formation of bakuchiol. In order to accept this proposal, it is necessary to substantiate the involvement of thiamine in irregular terpene biosynthesis. Thiamine was first suggested as a cofactor in squalene production by R. B. Woodward in 1961.[128,129] Also, when thiamine was excluded from the media of mold cultures and cell-free enzyme preparations, carotenoids[130-132] and squalene[133,134] were not synthesized. Yet upon addition of this

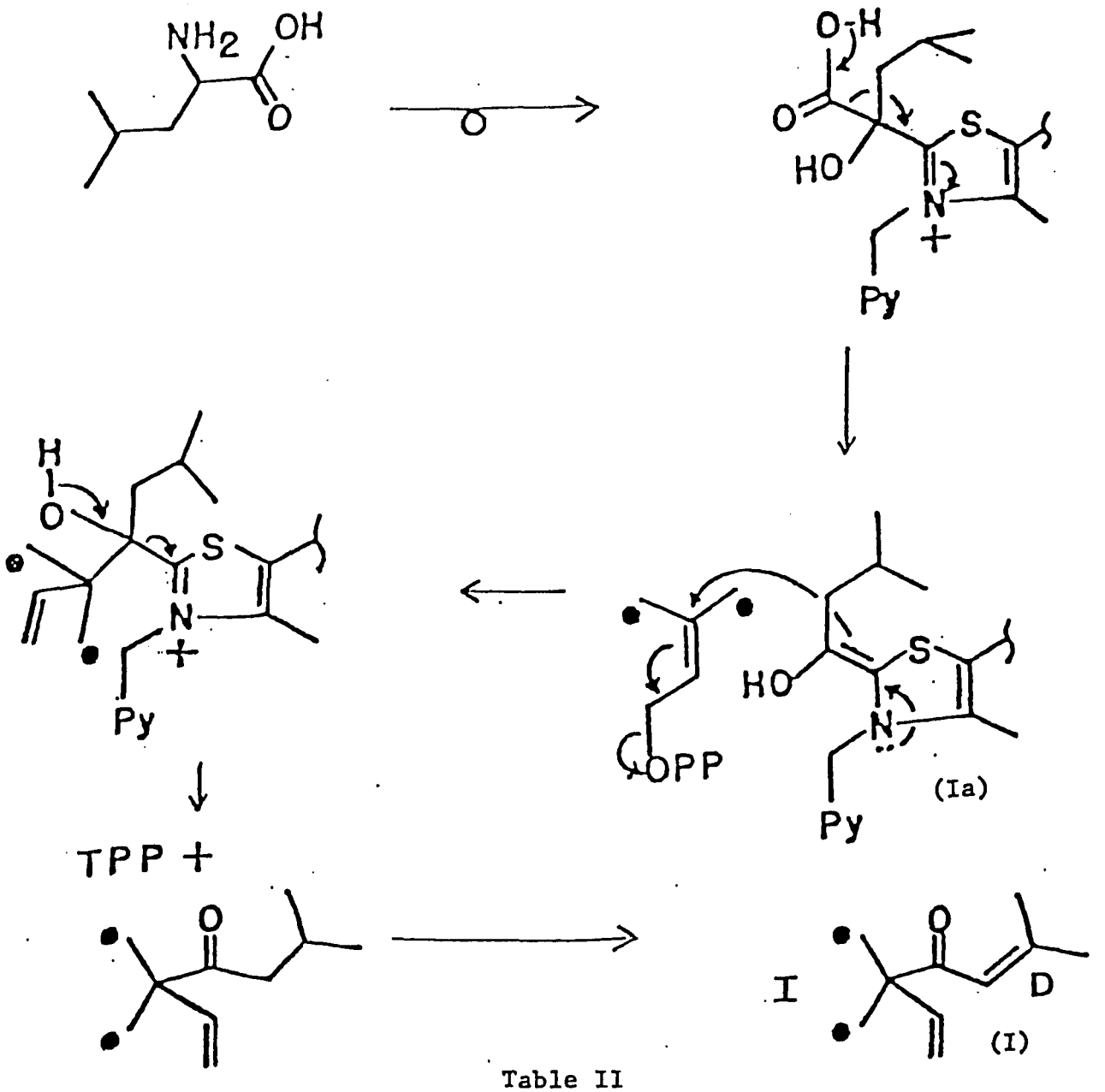



Table II

| Substrate | I | D % |
|---|---|---|
| 1. 3-methylbutanal | 93 | 6 |
| 2. α-hydroxyisocaproic acid | 89 | 7 |
| 3. α-hydroxyisocaproic acid and α-hydroxyisovaleric acid with MVA | 93 | 5 |

Figure 21. Proposed biosynthesis of artemisia ketone (I) via a thiamine adduct and incorporation of precursors of (Ia) into (I).

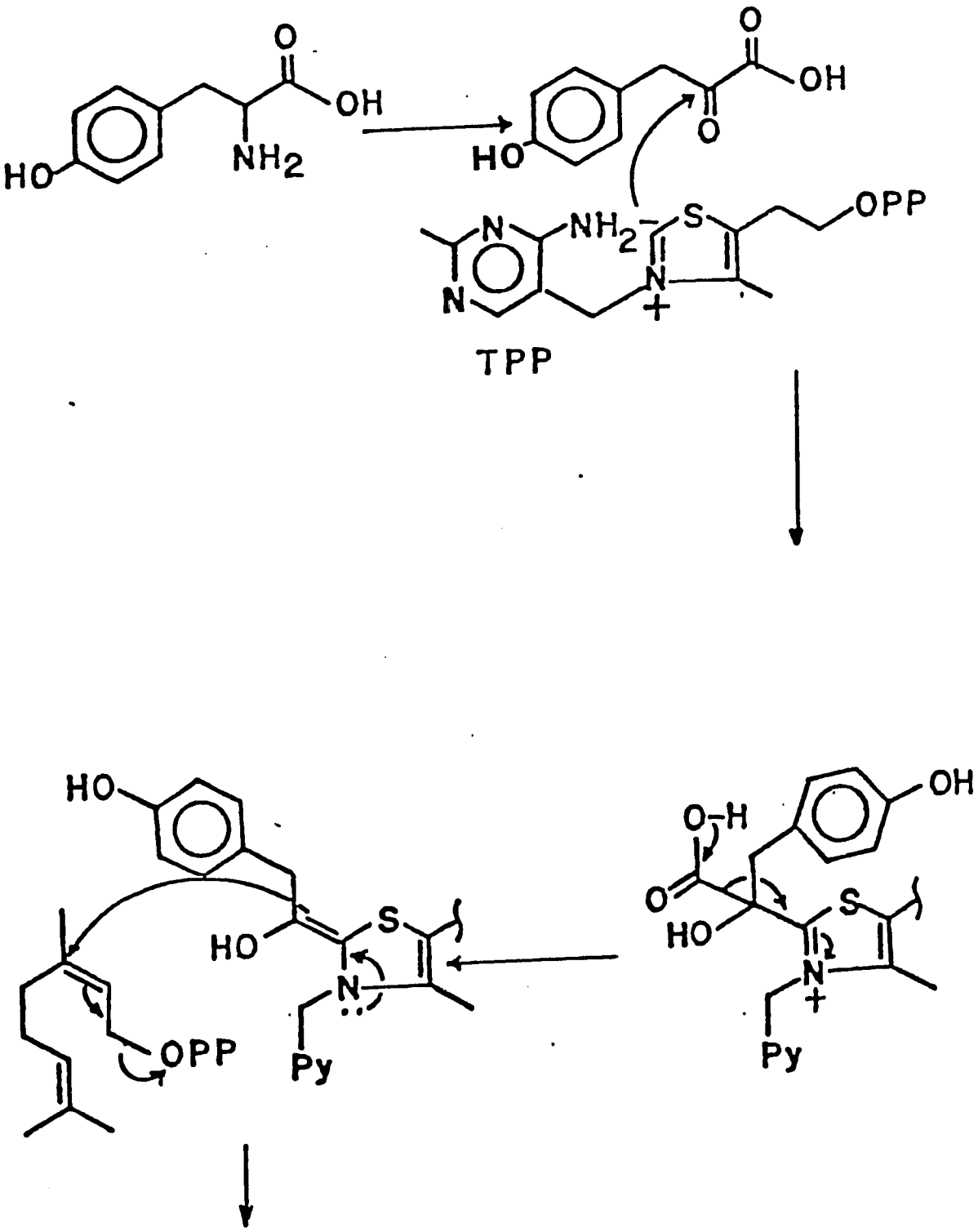
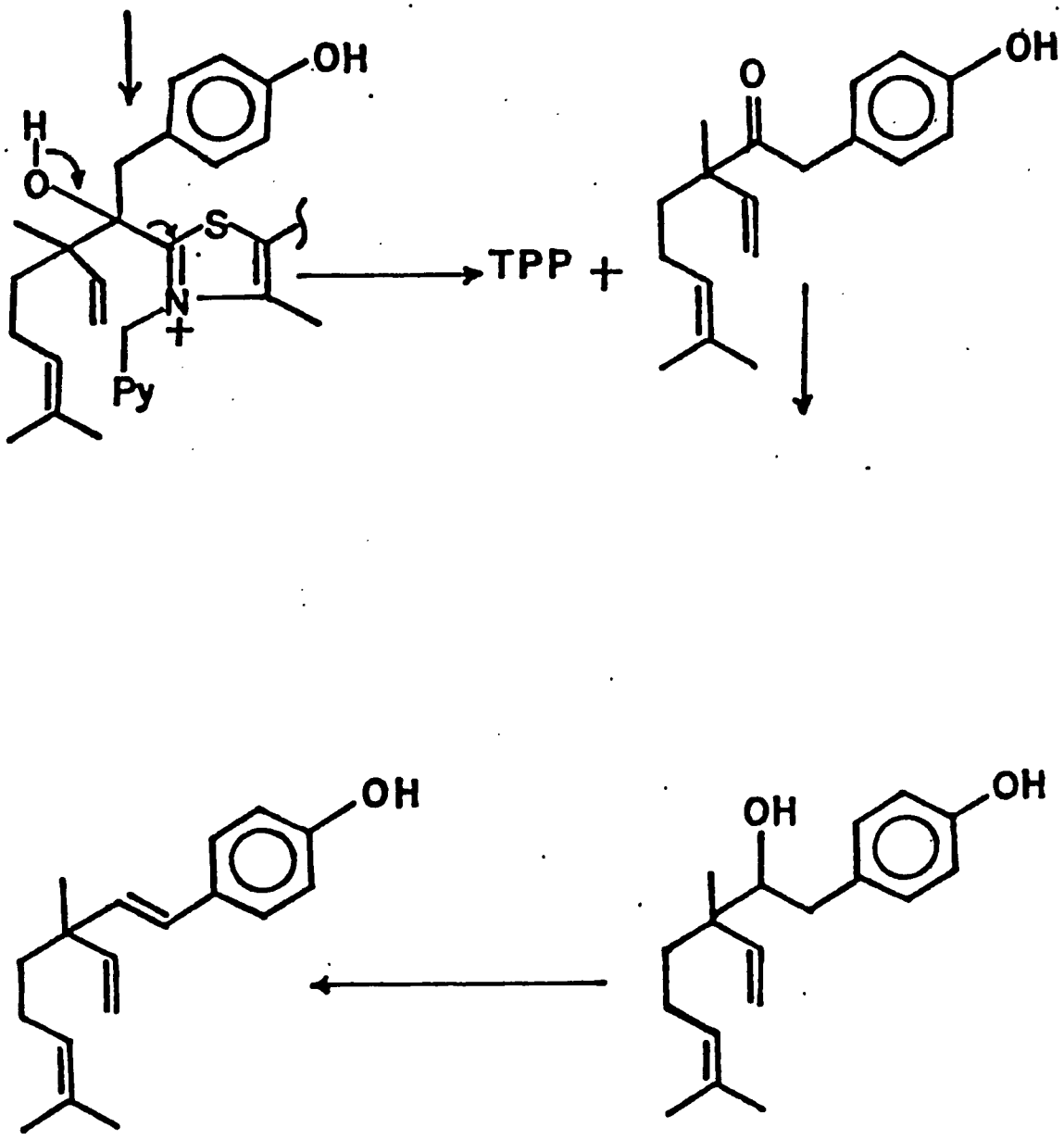



Figure 22. Proposed mechanism for the biosynthesis of bakuchiol.




Bakuchiol (II)

Figure 22. (cont.)

vitamin, biosyntheses commenced. Schotfer and Grobb[128] have shown that the mold *Phycomycis* can produce carotene when incubated with thiamine and ammonium lactate as its sole food source. Extensions of this work to irregular monoterpenes was undertaken by Bell[135] and later, by Karimian *et al.*,[125,126] who have shown that C-2 hydroxyalkyl derivatives of thiamine can serve as effective precursors of artemisia ketone analogues in cell-free enzyme systems. Based upon this work, the asymmetric labelling pattern found for artemisia ketone and the biogenetic considerations about bakuchiol, a similar theory concerning artemisia ketone was made.[125,126] Thus, 2-hydroxyisovaleryl thiamine attacks DMAPP in an $S_N2'$ fashion, followed by release of $B_1$. FAD oxidation of the dihydroartemisyl ketone completes the formation of this irregular terpene (Fig. 21). Other skeletons (e.g., chrysanthemyl, santolinyl and lavanulyl) may arise by isomerization of the corresponding alcohol to chrysanthemyl pyrophosphate. From this intermediate, the other terpene skeletons may be generated according to Bates' mechanism.[65]

## II. EXPERIMENTAL

General. The chemicals used in all experiments were considered sufficiently pure unless indicated otherwise. Thiamine chloride hydrochloride was purchased from Matheson, Coleman and Bell Manufacturing Chemists. Adenosine Triphosphate, Alkaline Phosphatase, and *p*-nitrophenyl phosphate were purchased from Sigma Biochemicals.

The TLC reference, 2,6,9,13-tetramethyl-6-vinyl-2,12-tetradecadien-7-one (compound 18a), and tritiated citral and geraniol were supplied by Dr. K. Karimian.

Quantum/Gram precoated plates (QGF) were used in thin layer chromatography (TLC). Matheson, Coleman, and Bell silica gel, 60-250 mesh (Grade 950); silica gel Woelm, Activity Grade I; and silica gel, 70-325 mesh (EM Reagents) were used in preparative layer chromatography. They are referred to as MCB, Woelm, and EM, respectively. Dry columns were eluted, using silica gel Woelm, Activity Grade III as a support. Aluminum oxide was purchased from M. Woelm and was Activity Grade I (cationotropic).

Melting points were determined with a melt-temp capillary melting point apparatus and are uncorrected. A Corning model 12 pH meter was used to determine the hydrogen ion concentration in the biological assays. In chemical preparations, pH paper (types

A and B) was supplied by Micro Essential Laboratory. Infrared spectra were determined on a Beckman IR-10 double beam spectrometer. $^{1}H$-NMR were recorded on a Varian A-60A spectrometer. All chemical shifts were reported in parts per million (δ-ppm) downfield from tetramethylsilane (TMS) when using organic solvents. HDO was considered an internal reference in aqueous media (δ 4.61). Mass spectral analyses were performed by Mr. Don Patterson on a Hewlett-Packard HP5985 mass spectrometer. Elemental analyses were performed by Mr. Ralph Seab of the Louisiana State University Chemistry Department.

High resolution mass spectrometry was performed at Florida State University by the Analytical Chemistry Department.

1. Synthesis of 2-(1-hydroxy-3,7-dimethyl-1-oct-6-enyl) thiamine chloride hydrochloride[126,136] 1.

Thiamine chloride hydrochloride (10.12 g; 30 mmol) and citronellal (5.4 ml; 60 mmol) were added to 250 ml of ethanol at 0°C. Sodium (1.38 g; 60 mmol) was dissolved in 100 ml of ethyl alcohol in a 250 ml addition funnel. The entire apparatus was flushed with nitrogen. The ethoxide solution was added dropwise and was complete in 15 minutes. After five hours of stirring, the solution was acidified with dry HCl gas. The suspension was filtered by suction and the precipitate was washed with 100 ml of solvent. The filtrate was concentrated to a small volume by flash evaporation and slowly added to a stirring ether solution (400 ml). The desired product precipitated at once. This

precipitate was filtered and washed with an equivalent volume of ether; Yield 9.1 g; 62%; M.P. 165-168°C (Lit,[126] 170-172°C) NMR 1.

2. Synthesis of 2-(1-hydroxy-3,7-dimethyloct-6-enyl)-4-methyl-5-(2-hydroxyethyl) thiazole[137] 2.

Sodium sulfite (8.45 g; 67.6 mmol) was dissolved in 50 ml of $H_2O$ and the pH was adjusted to 5 with 5 ml of 37% HCl. This solution and 1 (9.1 g; 18.6 mmol), dissolved in 100 ml of $H_2O$, were combined and stirred under nitrogen for 20 hours. The pH was then adjusted to 10 with 10% NaOH. The aqueous solution was extracted with two 50 ml portions of dichloromethane. The organic layers were combined, dried, filtered and concentrated under vacuum; Yield 4.47 g; 81%; NMR 2.

3. Reduction of citral to citronellal.

In an adaptation of the procedure of Adams,[138,139] citral (5 ml; 29.2 mmol) and 0.1 g of 10% Pd/C were added to 17 ml of 95% ethanol. The mixture was placed under approximately 2 atm. of $H_2$ on a Paar Hydrogenation apparatus until one equivalent of the gas was absorbed. The catalyst was filtered by passing the solution through a celite pad. The precipitate was then washed with two 20 ml portions of ethanol. TLC of the derived material showed 100% conversion to citronellal. Tritated citral was treated in the same manner in order to quantitatively obtain tritiated citronellal. Both the loss of the α vinyl signal and the presence of a triplet for the aldehyde proton in the NMR confirm this.

4. Synthesis of 2-(1-hydroxyethyl) thiamine chloride hydrochloride (HET)[126,136] 3.

Thiamine chloride hydrochloride (3.37 g; 10 mmol) was suspended in 100 ml of absolute ethanol at 0°C. Acetaldehyde (5 ml; 90 mmol) was added. A sodium ethoxide solution, prepared by the addition of sodium (460 mg; 20 mmol) in 50 ml of ethanol, was added over a 30 minute period. After five hours, the solution was acidified with dry HCl gas and filtered. The precipitate was washed with 100 ml of solvent. Concentration of the filtrate resulted in the formation of HET crystals; Yield 2.4 g; 63%; M.P. 232°C (Lit.[126] 234-236°C); NMR 3.

5. Reaction of HET with 5,5-dimethyl-1,3-cyclohexanedione (dimedone).

HET (1 g; 2.6 mmol), potassium carbonate (720 mg; 5.2 mmol), dimedone (740 mg; 5.2 mmol) and 5 drops of morpholine were all mixed in 50 ml of dimethylformamide and stirred under nitrogen for 20 hours. At this time, 50 ml of dichloromethane were added and the solid material filtered. The filtrate was concentrated by flash evaporation and the residue triturated with ether. TLC of the ether fraction in two solvent systems showed it to contain the acetaldehyde adduct of dimedone, 1,1-bis-(5,5-dimethyl-2-cyclohexan-1,3-dionyl)-ethane, 4, when compared to an authentic sample[140] (See Table III).

Table III

| Solvent | Rf 4 | Rf ether fraction |
|---|---|---|
| Ether | .73 | .75 |
| Benzene/methanol 20 : 1 | .33 | .31 |

6. Synthesis of 2-benzoyl-3-(2-methyl-4-amino-pyrimidin-5-yl) methyl-3a-methylperhydrofuro [2,3-d] thiazole 5.

Benzaldehyde (2 ml; 20 mmol), purified by the method described by Fieser and Fieser,[141] and thiamine chloride hydrochloride (3.37 g; 10 mmol) were added together in 100 ml of absolute ethanol at 0°C under a nitrogen atmosphere. The solution gradually turned brown. After five hours, 540 mg of ammonium chloride was added and the solution was stirred for 15 minutes at room temperature. The dark solution lightened to a yellow color. The solid material was filtered and the filtrate was concentrated under vacuum. The resulting gum was taken up into dichloromethane, filtered and concentrated. After repetition of this process, the gum was redissolved in a minimal amount of solvent and precipitated by its slow addition to ether; Yield 1.33 g; 35%; M.P. 163°C (Lit.[142] 168°C); NMR 4.

7. Attempted synthesis of 2-cinnamoyl-3-(2-methyl-4-amino-pyrimidin-5-yl) methyl-3a-methylperhydrofuro [2,3-d] thiazole 6.

The same process was repeated except freshly distilled cinnamaldehyde (2.5 ml; 20 mmol) was substituted for benzaldehyde. None of the desired compound was obtained upon work-up (as described in Experiment 6). An indefinite number of compounds were detected in the ether fraction by TLC which could not be chromatographically separated. This was due to the overlapping of spots forming a streak.

8. Synthesis of 3-methylbenzothiazolium hemisulfate 7.

Freshly distilled benzothiazole (5.4 ml; 50 mmol) and methyl sulfate (4.7 ml; 50 mmol) were stirred in 100 ml of toluene for 20 hours. The solid was filtered and washed with four 100 ml portions of petroleum ether; Yield 8.0 g; 80.4%; NMR 5.

9. Synthesis of 3-methylbenzothiazolinyl phenyl ketone 8.

Benzaldehyde (5.2 ml; 52 mmol), purified by the method of Fieser and Fieser,[141] and triethylamine (6.8 ml; 6.4 mmol) were dissolved in ethanol and brought to reflux. Compound 10 (8.27 g; 40 mmol) was added in small portions and the whole was refluxed for two hours after the final addition. The reaction mixture was concentrated under vacuum, and the residue was taken up into dichloromethane and extracted with water. The desired compound was crystallized from dichloromethane and petroleum ether; Yield 3.25 g; 32%; M.P. 134-137°C (Lit.[143] 140°C); NMR 6; IR 1.

10. Synthesis of benzanilide 9.

Benzoyl chloride (23.3 ml; 0.2 mol) was slowly added to a cooled solution of aniline (20 ml; 0.22 mol) and sodium carbonate (21.2 g; 0.2 mol) in 600 ml of benzene. The solution was warmed to room temperature and stirred for 24 hours. The mixture was filtered and washed exhaustively with acetone. Approximately 2 ml of aniline was added to the filtrate to react with any remaining benzoyl chloride. Successive crops of benzanilide were obtained by concentrating the solution by distillation and allowing the product to crystallize; Yield 34.3 g; 87%; M.P. 162°C (Lit.[144] 161°C); NMR 7; IR 2.

11. Synthesis of thiobenzanilide 10.

Benzanilide (34.3 g; 174 mmol) and phosphorus pentasulfide (43 g; 191 mmol) were reacted in 174 ml of pyridine in a modified procedure of Klingsberg and Papa.[145] The solution was refluxed for 40 minutes whereupon the resulting brown tar was poured on 300 g of crushed ice. Sodium hydroxide (7 g) was dissolved in a small amount of water and added. After the ice melted, the pH was adjusted to 7 by the addition of HCl. The precipitate was extracted in three successive washings with 100 ml portions of chloroform. After drying, the solvent was removed by flash evaporation. The desired compound was recrystallized from methanol and water; Yield 32.2 g; 87%; M.P. 98.5°C (Lit.[145] 96-96.5°C); NMR 8; IR 3.

12. Synthesis of S-phenacylthiobenzanilide 11.

Phenacylchloride (23.4 g; 151 mmol) and thiobenzanilide (32.2 g; 151 mmol) were dissolved in 250 ml of methanol. The solution was placed in an addition funnel and added dropwise to sodium methoxide solution (3.47 g of sodium and 100 ml of methanol). After three hours of stirring, the mixture was filtered; Yield 44.3 g; 89%. Recrystallization from ether and petroleum ether gave a product with a constant melting point of 78°C. NMR 9; IR 4.

Analysis for $C_{21}H_{17}NOS$

Calculated: C, 76.10; H, 5.17; N, 4.23

Found: C, 76.26; H, 5.25; N, 4.16

13. Attempted synthesis of 2,3,4-triphenyl-thiazolium chloride 12.

Thionyl chloride (140 mg; 2 mmol) and 11 (331 mg; 1 mmol) were stirred in 20 ml of benzene. At once, an oil formed at the bottom of the reaction vessel. Excess thionyl chloride was then added, which caused the oil to dissolve as HCl and $SO_2$ gases evolved. After stirring for two days, none of the desired thiazolium salt was formed. TLC indicated that only starting material was present.

14. Synthesis of nonanilide 13.[146]

Nonanoic acid (8.77 ml; 50 mmol) and thionyl chloride (3.60 ml; 55 mmol) were added together and stirred for 30 minutes. The solution was then diluted with 30 ml of benzene and refluxed for 15

minutes. After the heat was removed, aniline (9.11 ml; 100 mmol), diluted with 20 ml of benzene, was cautiously added and the whole stirred for another 15 minutes. The entire solution was poured onto 400 ml of crushed ice. After the ice melted, the benzene was decanted, extracted successively with water, 5% sodium hydroxide, 5% hydrochloric acid, and finally with water. The organic layer was dried and concentrated. The product was recrystallized from petroleum ether; Yield 6.13 g; 54%; M.P. 56-56.5°C (Lit.[147] 57.5°C); NMR 10; IR 5.

15. Synthesis of thiononanilide 14.

Phosphorus pentasulfide (9.6 g; 43.3 mmol) and 12 (9.32 g; 40 mmol) were dissolved in 40 ml of pyridine and refluxed for 40 minutes. The dark, viscous oil was poured onto an iced sodium hydroxide solution (1.8 g of sodium hydroxide dissolved in 50 ml of water and 400 ml of crushed ice). The pH was adjusted to 7 with dilute hydrochloric acid. The mixture was filtered after the ice melted and the precipitate washed with water. This solid was then dissolved in ether, dried, and passed through a short alumina column to remove the dark impurities. The eluant was extracted with water, dried, and crystallized; Yield 8.0 g; 80%; M.P. 41°C; NMR 11, IR 6, MS 1.

Analysis for $C_{15}H_{23}NS$

Calculated: C, 72.23; H, 9.30; N, 5.62

Found: C, 72.28; H, 9.21; N, 5.64

16. Synthesis of S-(p-chlorophenacyl)-thiononanilide 15.

Thioamide 17 (280 mg; 1 mmol) and *p*-chlorophenacyl bromide (228 mg; 1 mmol) were dissolved in 50 ml of dichloromethane. The reaction was stirred for two hours at which time 100 ml of petroleum ether were added. The resulting precipitate was collected and washed with 100 ml of petroleum ether and dried; Yield 450 mg; 93% (as the hydrobromide salt); NMR 12; IR 7; MS 2.

Mass (free base): $C_{23}H_{28}ClNOS$

Calculated ($^{35}Cl$): 401.1580

Found: 401.1579

17. Attempted synthesis of 2-octyl-3-phenyl-4-(4-chlorophenyl) thiazolium chloride 16.

A solution of 14 (480 mg; 1 mmol) and thionyl chloride (0.14 ml; 2 mmol) was stirred in 20 ml of benzene. At once, the solution turned to a dark purple color. Although the color persisted for the entire course of the reaction (two days), none of the desired compound was detected by chromatographic techniques. TLC and dry column chromatography resulted in the detection of the starting compound, 14, as the sole material present.

18. Preparation of geranyl bromide[148]

Phosphorus tribromide (22 ml; 232 mmol) was placed in a 500 ml round bottom at 5°C. A solution of linalool (100 ml; 559 mmol) and pyridine (12.5 ml; 155 mmol) was added dropwise to the $PBr_3$. The mixture was stirred for one hour, at which time the

temperature was raised to 25°C and stirred for 16 hours. The reaction was terminated by pouring the solution into 100 ml of a saturated sodium bicarbonate solution in 600 ml of crushed ice. The aqueous phase was stirred vigorously during the process. After the ice had melted, the organic layer was taken up into ether, washed with 5% sulfuric acid and dried. The solvent was then removed under vacuum. Geranyl bromide is best stored in this condition as it decomposes rapidly after distillation. Hence, aliquots are distilled right before use. NMR 13.

19. Synthesis of 3,6,9,13-tetramethyl-9-vinyl-tetradeca-3,7,12-trien-6-ol 17.

A Continuous Flow Reformatsky Column was prepared according to the procedure of Ruppert and White.[149,150] An addition funnel was attached to a 60 ml column fitted with a glass wool plug. This apparatus was placed on a three-neck round bottom and drying tubes were used to keep the internal atmosphere dry. The apparatus is shown in Figure 23. The glass was flamed with a Bunsen burner and the column was wrapped with heating tape.

Approximately 50 g of zinc metal (20 mesh) was activated by successive washings with 100 ml of: 5% HCl, ethanol, acetone, and ether. The zinc was dried under vacuum (1 mm Hg) and placed in the 60 ml reaction column. Tetrahydrofuran, dried with 4 Å molecular sieves, was added to the column and brought to a gentle reflux. Some solvent was allowed to pass through so that a head of one inch was established.

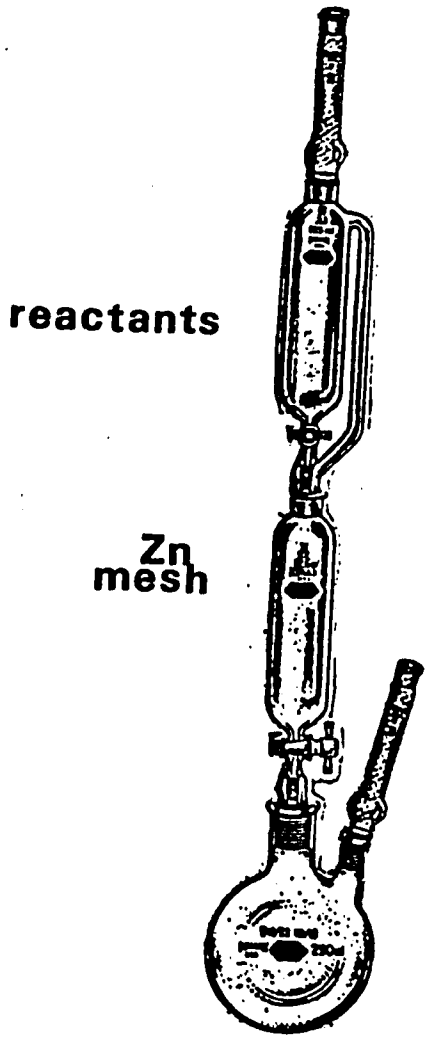



Figure 23. The Continuous Flow Reformatsky Column.

To obtain the desired compound, freshly distilled geranyl bromide (10 ml; 50 mmol) and citral (4.3 ml; 25 mmol) were diluted with 50 ml of THF and placed in the addition funnel. A flow rate of one drop per second was initiated in order to keep the solvent volume in the column constant. Upon final addition of this mixture, the column was washed with 50 ml of THF. The organic solution was poured into a single-neck round bottom with 5 ml of water. Upon solvent removal, the syrup was dissolved in 50 ml of petroleum ether. Successive extractions with: 50 ml of 10% $H_2SO_4$, 30 ml of 10% NaOH, and 100 ml of water were followed by drying and solvent removal. The resulting oil was distilled at 1 mm Hg; the fraction boiling at 125-155°C was collected. NMR of the syrup showed it to be identical to that obtained by Karimian.[126] NMR 14.

20. Attempted synthesis of 2,6,9,13-tetramethyl-6-vinyl-tetradeca-2,8,12-trien-7-one 18.

Fieser and Fieser's[151] chromic acid solution was prepared by the following method: Chromium trioxide (2.67 g), $H_2SO_4$ (2.3 ml) and water (10 ml) were cautiously mixed with cooling. In 100 ml of acetone, 22 (290 mg; 1 mmol) was dissolved and cooled to 5°C. The chromic acid solution was added until a faint orange color persisted (approximately 0.3 ml). A small amount of sodium carbonate and sodium sulfite was added to destroy the acid and excess oxidizing agent. The acetone was decanted and the residue triturated with 50 ml of acetone. The organic fractions were

combined and the solvent removed under reduced pressure. Failure of the expected upfield singlet, characteristic of the angular methyl (δ 1.2), to appear and no carbonyl absorption in the IR spectrum indicated that the reaction failed.

21. Synthesis of 1-phenyl-2,6-dimethyl-2-vinyl-hept-5-en-1-one 19.

A Continuous Flow Reformatsky Column was prepared as previously described. Benzoyl chloride (1.75 ml; 15 mmol) and geranyl bromide (8.9 ml; 45 mmol) were mixed in 50 ml of THF and added to the dropping funnel. Upon completion of the column and work-up, the syrup was purified on a 15 inch by 1 inch silica gel column (MCB) using petroleum ether/ether (5:1) as the eluting solvent; Yield 1.96 g; 54%; NMR 15; IR 8; MS 3.

Analysis for $C_{17}H_{22}O$

Calculated: C, 84.25; H, 9.15

Found: C, 84.34; H, 9.54

22. Synthesis of 4-chlorobutyl benzoate[156] 20.

Benzoyl chloride (3.14 ml; 27 mmol), THF (2.82 ml; 34.7 mmol), and 0.5 g of freshly fused zinc chloride were added sequentially to a 100 ml round bottom. After the initial reaction subsided, the vessel was warmed to 100°C and allowed to cool. Sodium carbonate (1.4 g; 13.2 mmol) was added with 70 ml of benzene and the whole was stirred for one hour. The liquid was decanted and the solvent removed by flash evaporation. NMR 16.

23. Preparation of geranic acid 21.

In a scrupulously clean round bottom, silver nitrate (17 g; 0.1 mol) was dissolved in 100 ml of water. Citral (8.1 ml; 50 mmol) in 25 ml of THF was added. Ten grams of sodium hydroxide, dissolved in 75 ml of water was poured into the reaction with vigorous stirring. The resulting stirred suspension was kept under nitrogen for 16 hours. Silver was filtered off and the filtrate was acidified to pH 3. Geranic acid, separated from the aqueous layer, was taken up in 100 ml of petroleum ether and decanted. The remaining liquid was extracted with 20 ml of petroleum ether. The organic layers were combined, dried, and concentrated by flash evaporation. Distillation at reduced pressure (1 mm Hg) afforded geranic acid (B.P. 119°C); Yield 6.0 g; 71%; NMR 17.

24. Synthesis of 18 using geranic acid.

The following reagents were combined in 20 ml of benzene: geranic acid (840 mg; 5 mmol), pyridine (0.8 ml; 10 mmol), thionyl chloride (0.4 ml; 5.5 mmol), and four drops of DMF. The solution was refluxed for one hour at which time the hydrochloride was filtered. Solvent and unreacted thionyl chloride were removed by flash evaporation; pyridine was evacuated at 1 mm Hg. The resulting geranoyl chloride and freshly distilled geranyl bromide (3 ml; 15 mmol) were diluted with 25 ml of THF and placed in an addition funnel of the Continuous Flow Reformatsky Column.

The preparation is described in Experiment 19. After the work-up procedure, the oil was chromatographed on a 12 inch by 1 inch silica gel column (Woelm), using 5% ether in petroleum ether (Rf 0.81). Microdistillation of the collected fractions afforded the desired compound; Yield 500 mg; 31%; NMR 18; IR 9; MS 4.

Molecular weight: $C_{20}H_{32}O$

Calculated: 288.2453

Found: 288.2462

An additional fraction (Rf 0.65) was obtained and was shown to be 4-chlorobutyl geranate (NMR 19).

25. Synthesis of 2-(3,7-dimethyl-1-oct-6-en-1-onyl)-4-methyl-5-(2-hydroxyethyl) thiazole 22.

Manganese dioxide, prepared by the method of Sondheimer et al.,[153,154] was used as the oxidizing agent in a modification of the procedure described by Papadopoulous.[155] Seven grams of this reagent and 2 (1 g; 3.4 mmol) were added together in 50 ml of dichloromethane and refluxed for five hours under a blanket of nitrogen. The reaction was filtered over celite and washed until clear. Concentration of the solution afforded the product; Yield 810 mg; 81%; NMR 20.

26. Synthesis of 2-[1-hydroxy-1-(2',6'-dimethyl-5'-heptenyl)-2,6-dimethyl-2-vinyl-5-heptenyl]-4-methyl-5-(2-hydroxyethyl) thiazole 23.

A Continuous Flow Reformatsky Column was prepared as

previously described. Geranyl bromide (1.2 ml; 6 mmol) and 22 (810 mg; 2.8 mmol), diluted with 50 ml of THF, were allowed to react on the column. Work-up was performed in the usual manner. The syrup was purified on a 12 inch by 1 inch dry column using ether as a solvent. The fractions containing 23 also contained 2,6-dimethyl-2,6-octadiene, a by product of the Reformatsky reaction. The fractions were combined and the solvent removed under reduced pressure. The oil was taken up in a small amount of petroleum ether and applied to a short-path dry column (3 inches by 3/4 inch) and eluted with the same solvent until all the alkene was removed. Ether was then applied to remove the desired compound. Concentration of this fraction yielded 180 mg; 15% yield. NMR 21; IR 10; MS 5.

Molecular weight: $C_{20}H_{43}NO_2S$

Calculated: 433.3014

Found: 433.2989

27. Synthesis of farnesyl bromide[148] 24.

Nerolidol (11.5 ml; 45 mmol) and pyridine (1.2 ml; 12.6 mmol) were cooled to -4°C. Phosphorus tribromide (1.8 ml; 18.9 mmol) was added dropwise. The reaction was stirred for 18 hours at room temperature. At this time, the reaction was worked up as described in experiment 18. Distillation at 3 mm Hg afforded the desired compound; Yield 8.3 g; 65%; NMR 22.

28. Synthesis of 2,6,9,13,17-pentamethyl-9-vinyl-octadeca-2,11,16-trien-8-ol 25.

A Continuous Flow Reformatsky Column was prepared as previously described. Freshly distilled citronellal (5.3 ml; 29.3 mmol) and farnesyl bromide (8.3 g; 29.3 mmol) were diluted in 100 ml of THF and added to the column. After work-up, the oil was chromatographed on a 12 inch by 1 inch silica gel column (EM) using 5% ether in petroleum ether as a solvent; Yield 2.8 g; 26%; NMR 23; IR 11; MS 6.

Molecular weight: $C_{25}H_{44}O$

Calculated: 360.3392

Found: 360.3395

29. Synthesis of 2,6,9,13,17-pentamethyl-9-vinyl-octadeca-2,11,16-trien-8-one 26.

Oxidation of 25 was achieved by the procedure of Radcliffe.[156] Chromium trioxide (5.15 g; 51.5 mmol) and pyridine (8.34 ml; 103 mmol) were dissolved in 200 ml of dichloromethane at 0°C. Alcohol 25 (3.08 g; 8 mmol) was diluted in 50 ml of dichloromethane and added to the chromate solution. The resulting solution was stirred for 20 minutes and terminated by the addition of 100 ml of 30% sodium hydroxide. The layers were separated and the aqueous layer was extracted with 250 ml of dichloromethane. After the organic layers were combined, successive extractions with 5% HCl and brine were performed. The dichloromethane was removed and the oil

chromatographed using 5% ether in petroleum ether on a 12 inch by 1 inch silica gel column (MCB); Yield 2.17 g; 74%; NMR 24; IR 12; MS 7.

Analysis for $C_{25}H_{42}O$

Calculated: C, 83.73; H, 11.79

Found: C, 83.93; H, 12.01

30. Biosynthesis of 1,10-disopentenyl-3,4-dihydro-artemesia ketone in cell-free yeast preparations using $^{3}H$-geraniol and 1.[157,158]

Fleishmann's "Active Dry" Yeast (34 g) was mixed with fine glass beads in 120 ml of 0.066 M $(NH_4)_2HPO_4$ solution and pulverized with a mortar and pestle. The slurry was incubated for three hours at 37°C and then centrifuged at 15,000 rpm for one hour. The supernatant (75 ml) was decanted onto 33.8 g of $(NH_4)_2SO_4$. The solution was cooled to 0°C. Precipitation was complete upon final dissolution of the solid ammonium sulfate. The cloudy suspension was centrifuged for 15 minutes at 4,000 rpm and the liquid discarded. The solid enzymes were resuspended in 15 ml of 0.06 sodium phosphate buffer. The following compounds were added sequentially:

a. 0.3 g (1.2 mmol) $MgSO_4 \cdot 7H_2O$

b. 0.76 g (1.3 mmol) ATP : 1.5 $H_2O$

c. 0.4 g (1.3 mmol) glutathione

d. 4 drops Triton X-100

e. 0.06 ml (0.3 mmol) geraniol

f. 0.15 g (0.3 mmol) ($^{3}$H)1 in 5 ml $H_2O$ and 0.09 g $Na_3PO_4$

total dpm: 13,540

After incubation at 37°C for three hours, 10 ml of a 20% potassium hydroxide in methanol solution was added and the whole was heated at 68°C for one hour. The precipitated enzymes were centrifuged at 15,000 rpm for 15 minutes and the supernatant decanted. This liquid was extracted with seven 10 ml portions of ether. The protein pellet was extracted by adding 50 ml of boiling acetone and filtering. The precipitate was washed with 30 ml of hot acetone. Ether and acetone layers were concentrated under vacuum and the residue taken up in 5 ml of dichloromethane.

One milliliter was streaked on a thin layer chromatography plate (Quantum 1 Gram) and eluted with an ether/petroleum ether (5:1) solvent system against an authentic sample.[126] After the corresponding fraction was scrapped off the plate, the silica gel was boiled in 50 ml of acetone. This process was repeated. Upon concentration the oil was taken up in a toluene-based scintillation fluid[159] (prepared by dissolving 4.9 g of "PPO," 2,5-diphenyl oxazole, and 0.1 g "POPOP," 1,4 bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 liter of toluene). Total disintegrations found; 720 dpm, which corresponds to 3,600 dpm total, or 7.4% incorporation.

31. Biosynthesis of 26 using ($^3$H) 1 and farnesol in a cell-free yeast enzyme preparation.

Fleishmann's Yeast (68 g) was mixed in 196 ml of a 0.66 M $(NH_4)_2HPO_4$ solution by pulverizing the solution with glass beads in a Waring blender for five minutes as outlined in the previous experiment, to yield 100 ml of supernatant. Ammonium sulfate (45 g) was added after the solution was chilled in an ice bath. Stirring the liquid for 30 minutes resulted in the complete precipitation of the required enzymes, which were pelleted by centrifugation. The solid enzyme was resuspended in 15 ml of a sodium phosphate buffer (pH 7.2). The following compounds were dissolved in 10 ml of the phosphate buffer:

a. 0.3 g (1.2 mmol) $MgSO_4 \cdot 7H_2O$
b. 0.7 g (1.2 mmol) $ATP \cdot 1.5H_2O$
c. 0.4 g (1.3 mmol) glutathione
d. 0.15 g (1.2 mmol) ($^3$H)1; 4000 cpm

This solution was added to the enzyme mixture, followed by four drops of Triton X-100 and farnesol (0.08 ml; 1.2 mmol). The enzyme preparation was incubated at 35°C for three hours, after which work-up proceeded in the usual manner. The obtained oil was chromatographed on a thin-layer plate against an authentic sample of 26 (experiment 29) in petroleum ether/ether (10:1). The corresponding band was removed and the desired compound was removed (see experiment 30). This material showed an activity of 300 cpm or 7.5% incorporation.

The above experiment was repeated except farnesol was withheld so that the enzyme preparation and all other components could pre-incubate for two hours. Farnesol (0.08 ml; 1.2 mmol) was then added and the incubation proceeded for another three hours. After work-up and chromatography no discernable difference in the amount of incorporation was found.

32. Attempted biosynthesis of 23 using $^{3}$H-geraniol and 2 in the cell-free yeast enzyme preparation.

Fleishmann's Yeast (68 g) is prepared as described in the previous experiment. Upon final precipitation of the desired enzyme, the following components are added in 30 ml of the phosphate buffer and brought to pH 7.2 by the use of 10% NaOH:

a. 0.59 g (2.4 mmol) $MgSO_4 \cdot 7H_2O$

b. 1.32 g (2.4 mmol) ATP (sodium salt)$\cdot 1.5H_2O$

c. 0.74 g (2.4 mmol) glutathione

The precipitated enzymes were suspended in this solution. Subsequently, the following were added:

d. 0.11 ml (0.6 mmol) $^{3}$H-geraniol; 343,000 cpm

e. 0.18 g (0.6 mmol) 2 in 0.5 ml of ethanol

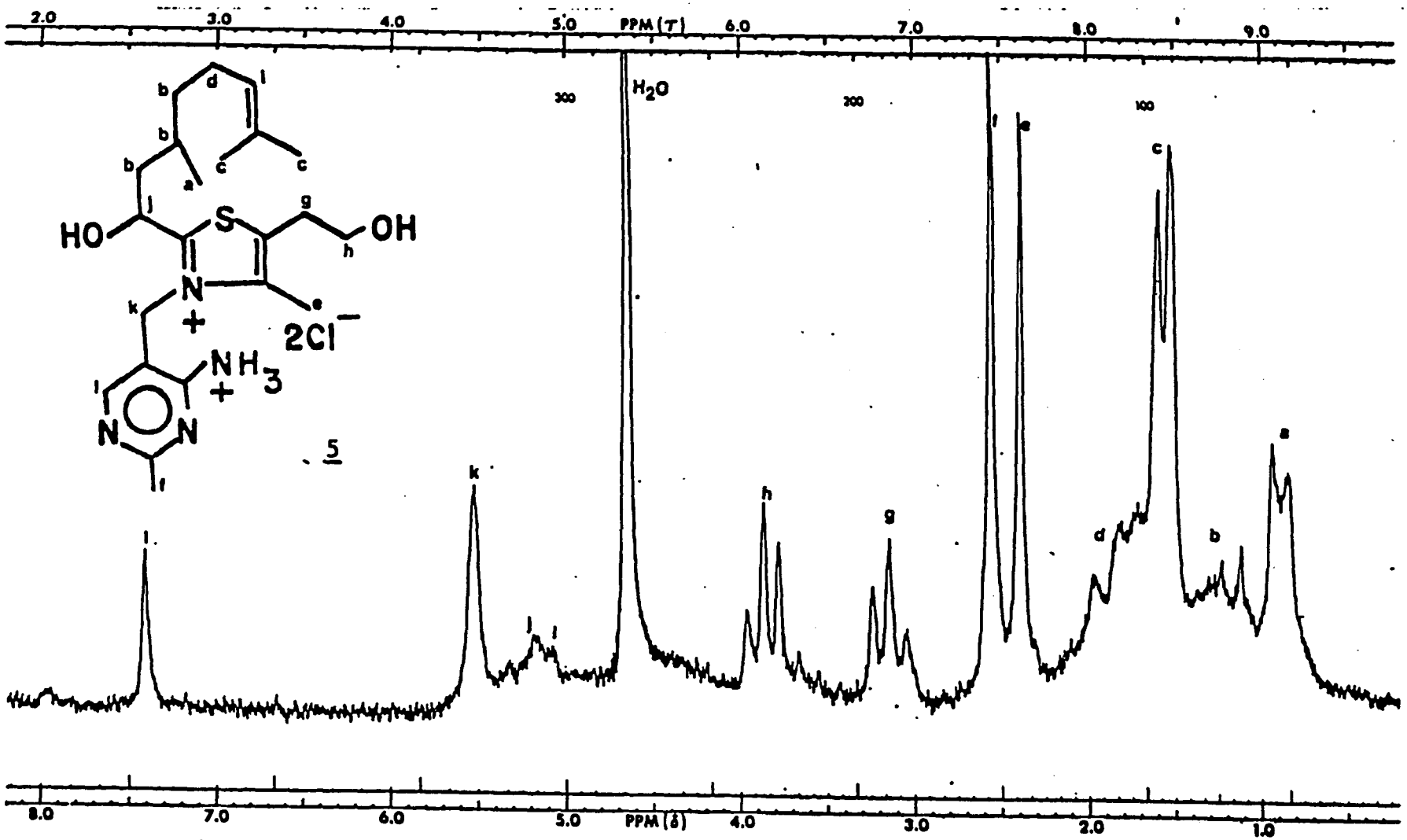
f. 4 drops Tritox X-100

After three hours of incubation 100 mg (Activity* 460 units/mg) of "calf mucosa" alkaline phosphatase was added and incubation continued for another hour. Work-up was achieved in the usual manner. However, upon chromatographing the obtained oil against

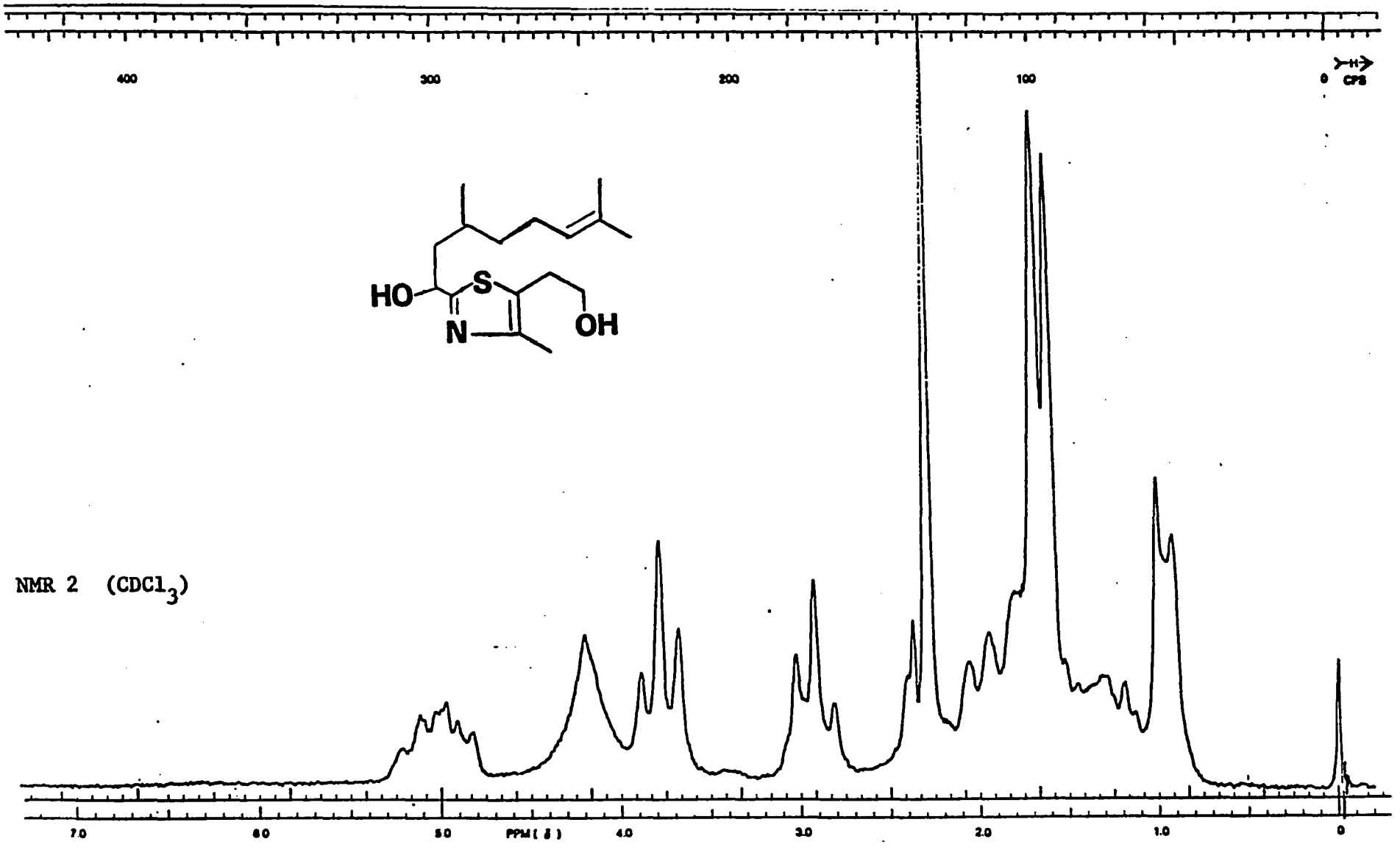
an authentic sample of 23 in ether and in benzene/methanol (40:1) none of the desired product would be detected.

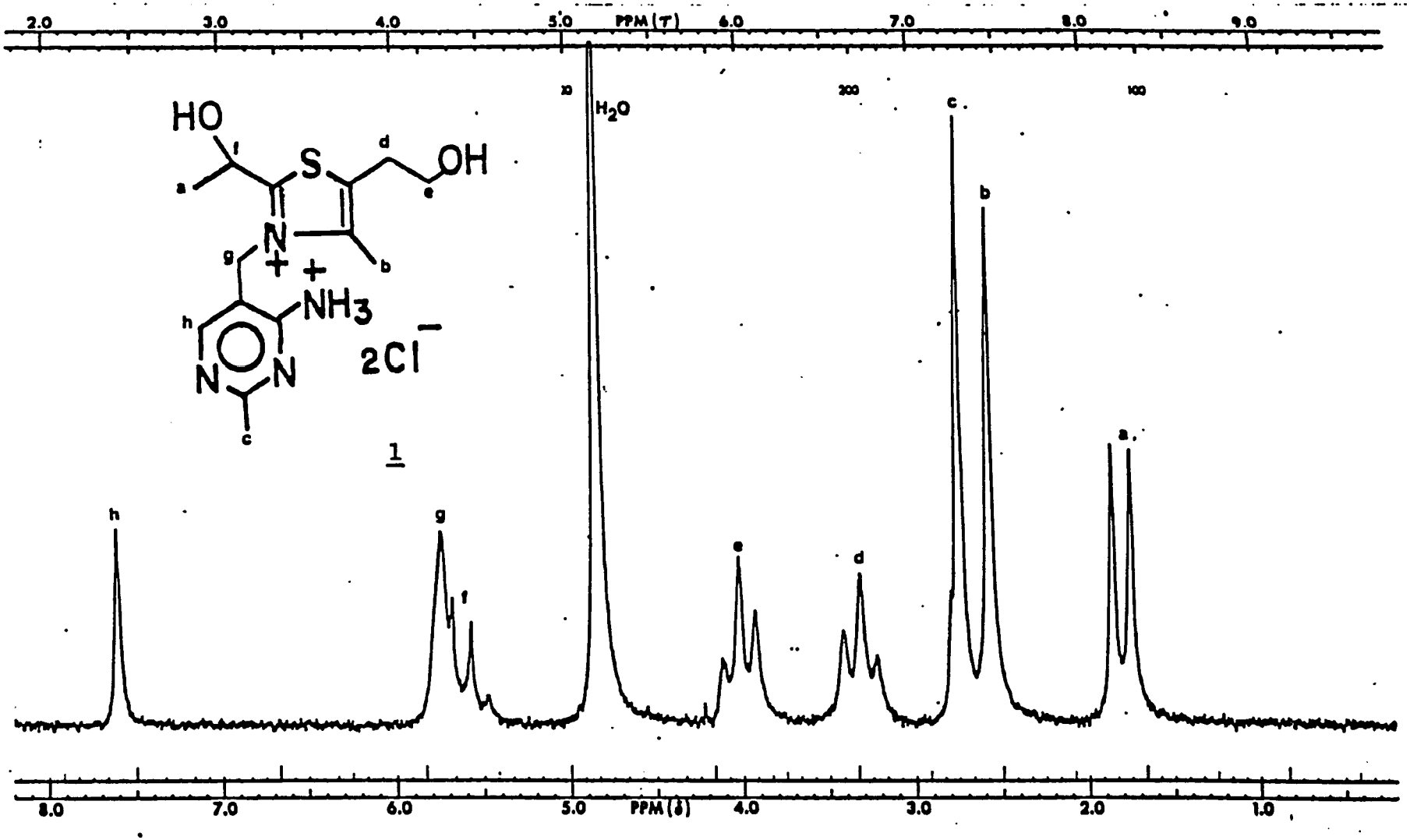
---

*One Activity unit is defined[160] as the amount of enzyme required to cause the change of one optical density (OD) unit/min. of a 0.01 M PNPP solution (in 0.6 M Tris-hydroxy methyl amino methane (Tris) buffer, pH 8.2). For this determination 100 mg of alkaline phosphate was dissolved in 100 ml of a 0.01 M Tris buffer (pH 7.5) containing 0.001 M $MgCl_2$. In order to do the assay 0.3 ml of a 1/10 strength enzyme solution and 1.7 ml of the PNPP solution were mixed. The rate was derived from the linear phase of the reaction.


2.0
3.0
4.0
5.0
PPM (τ)
6.0
7.0
8.0
9.0
H2O
300
200
100
HO
OH
S
N
+
2Cl⁻
NH3
+
N
N
5
k
h
g
d
c
b
a
l
j
i
f
e
8.0
7.0
6.0
5.0
PPM (δ)
4.0
3.0
2.0
1.0

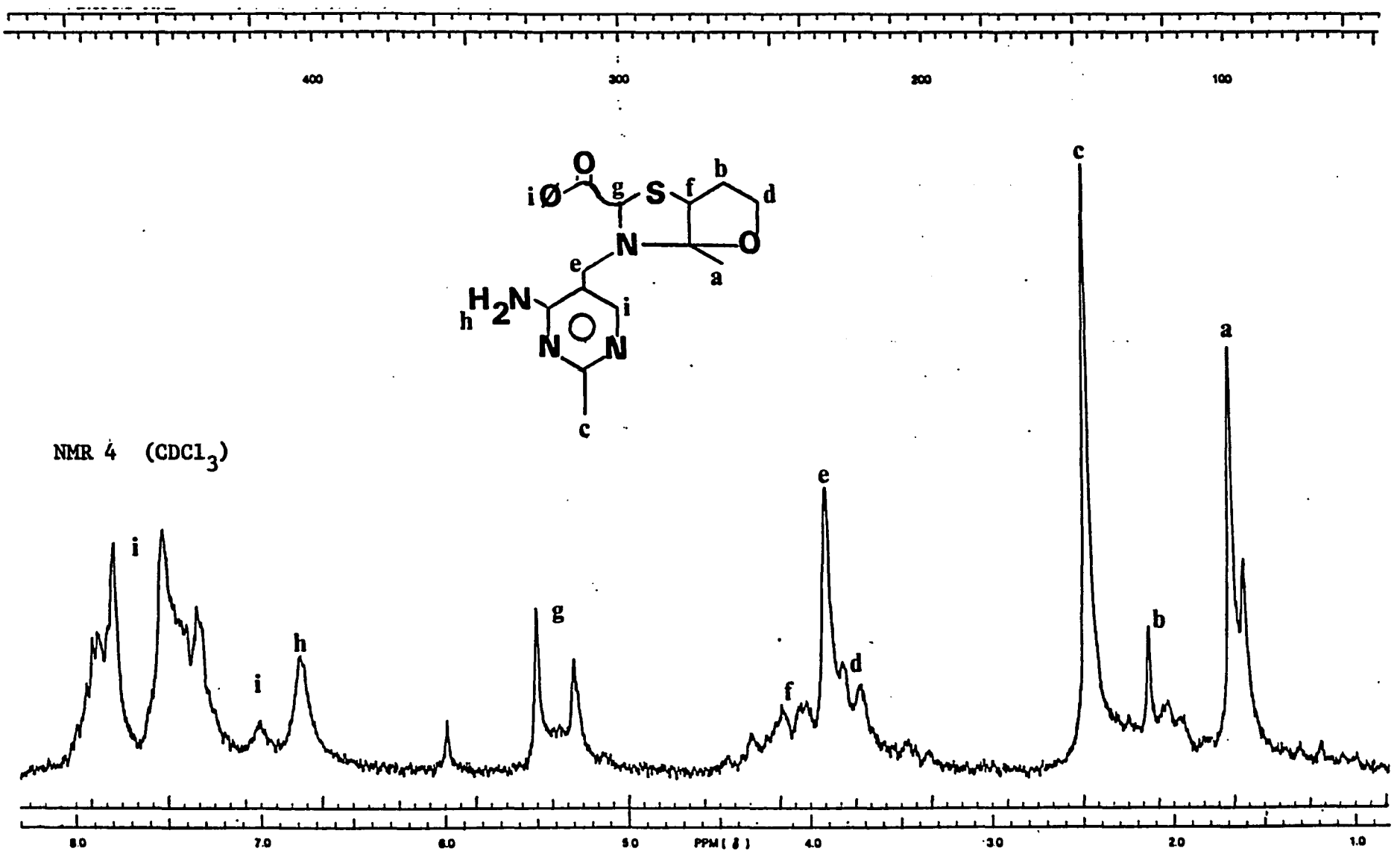
NMR 1 ($D_2O$)

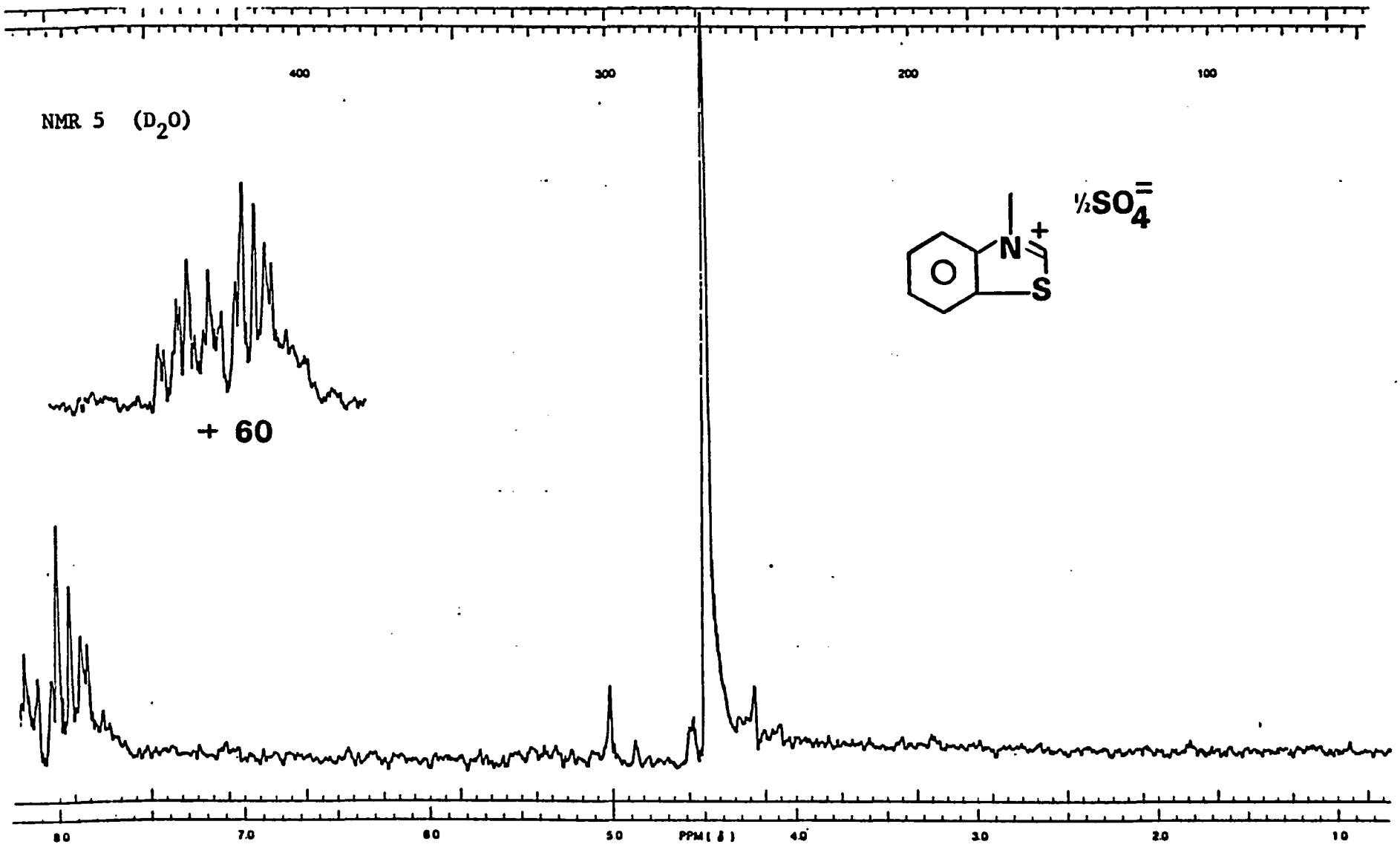

400
300
200
100
0
CPS
HO
S
N
OH
NMR 2 (CDCl3)
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0
0

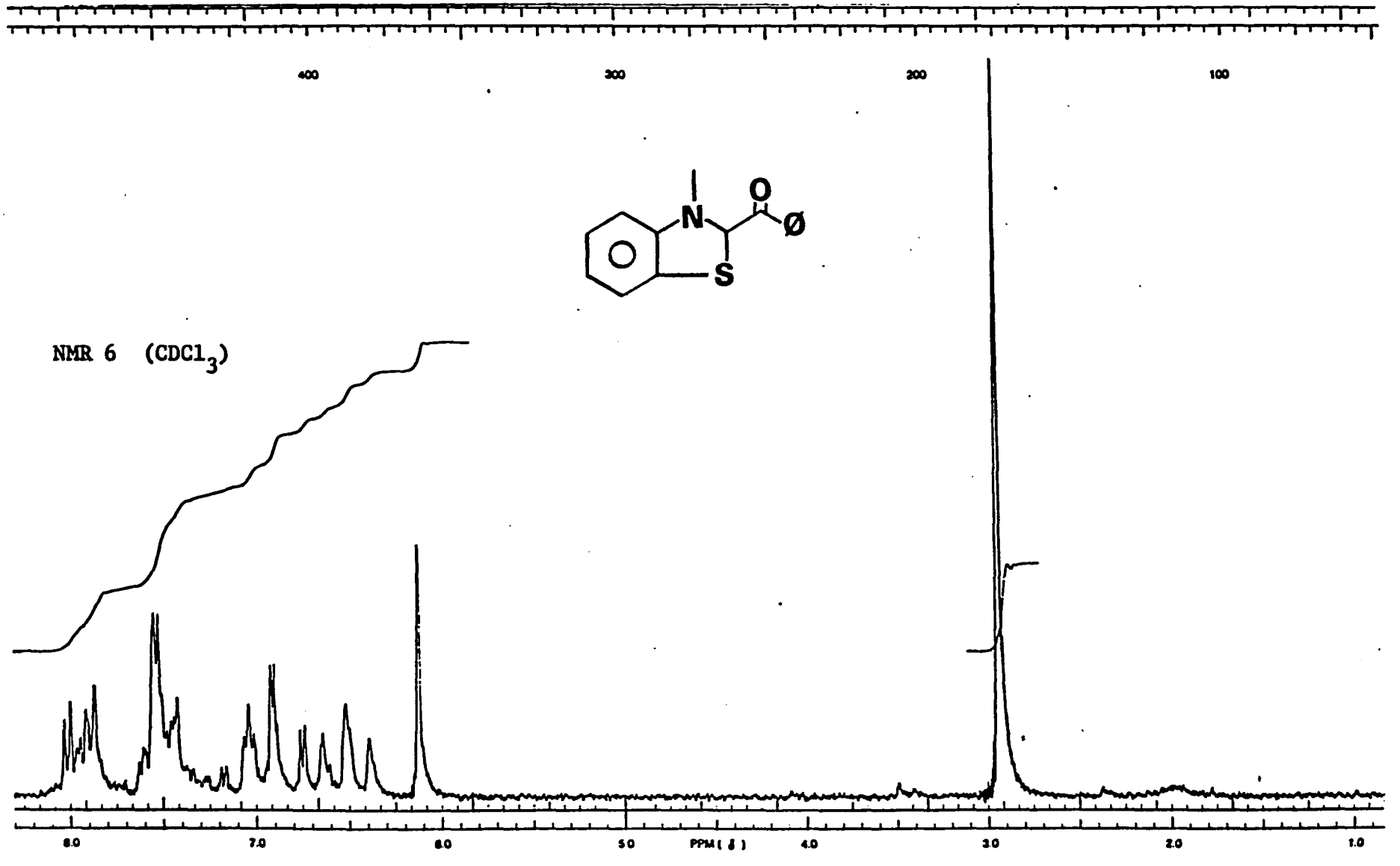



1

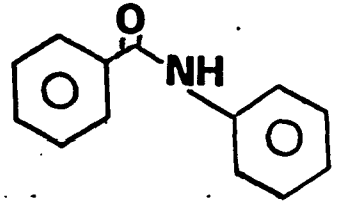
NMR 3 ($D_2O$)

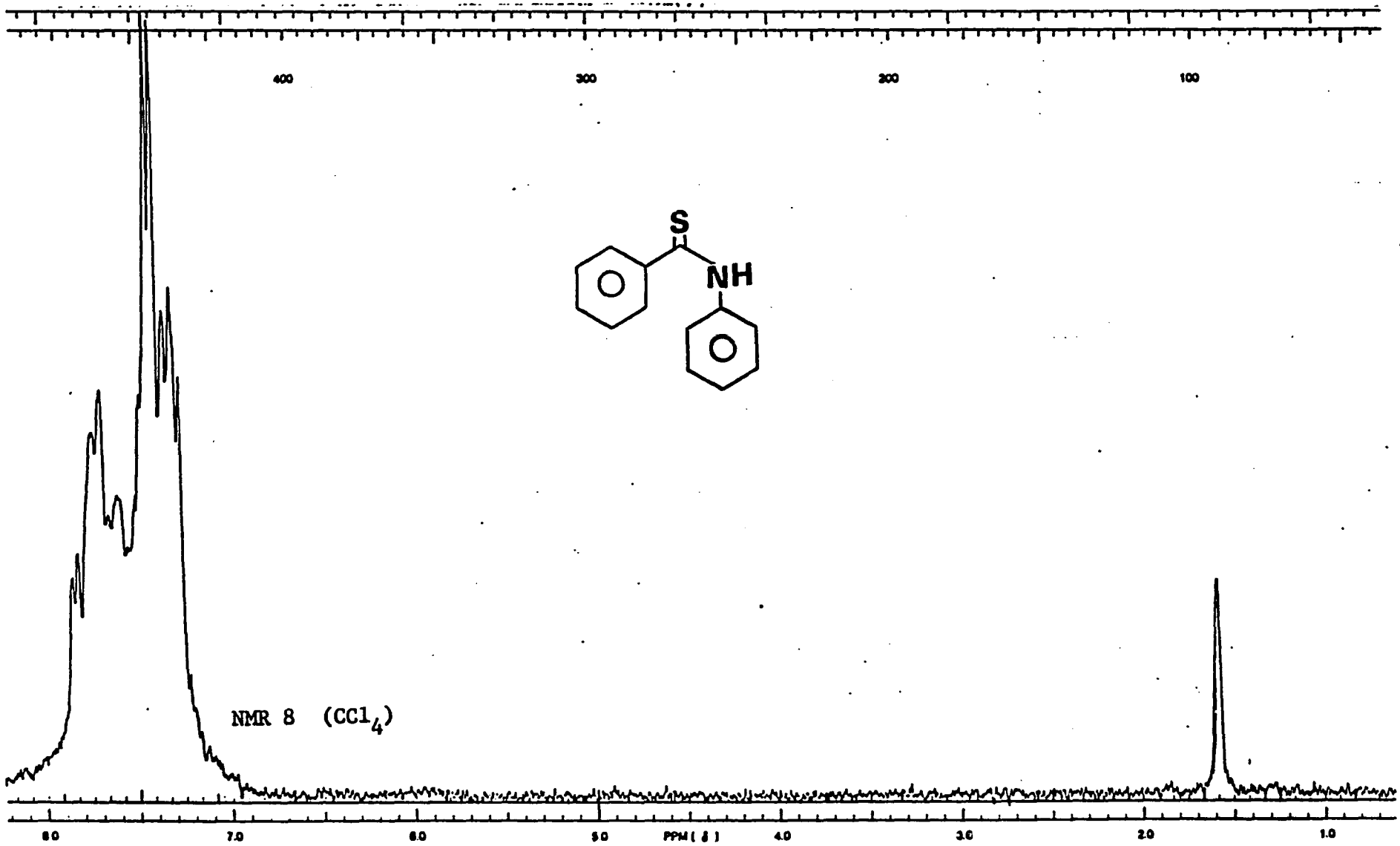

400
300
200
100
O
iØ
g
S
f
b
d
N
O
a
e
$H_2N$
h
i
N
N
c
NMR 4 ($CDCl_3$)
i
i
h
g
f
e
d
c
b
a
8.0
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0

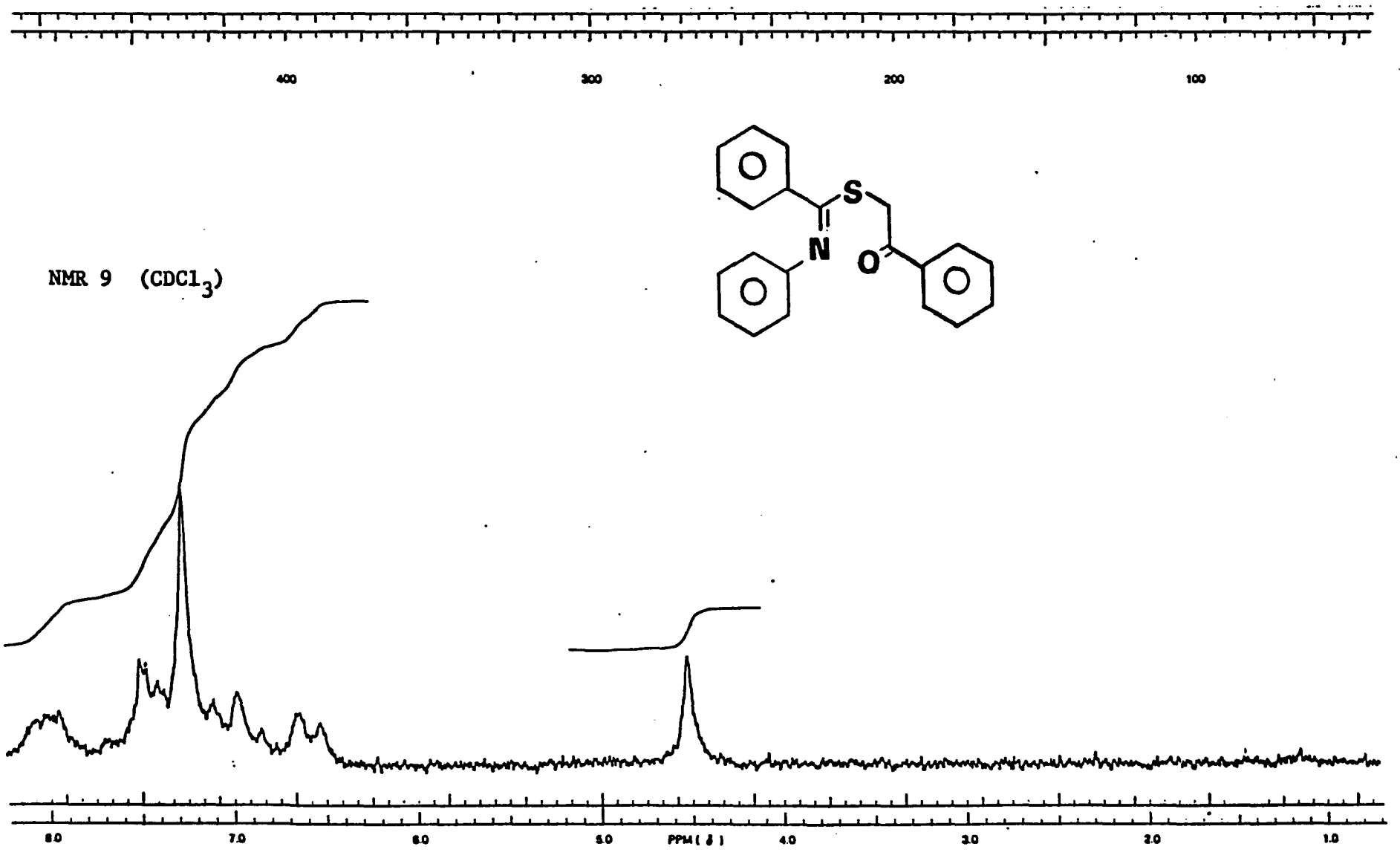

NMR 5 ($D_2O$)
+ 60
½$SO_4^=$
N
S
O
400
300
200
100
8.0
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0


400
300
200
100
O
N
Ø
S
NMR 6 (CDCl3)
8.0
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0

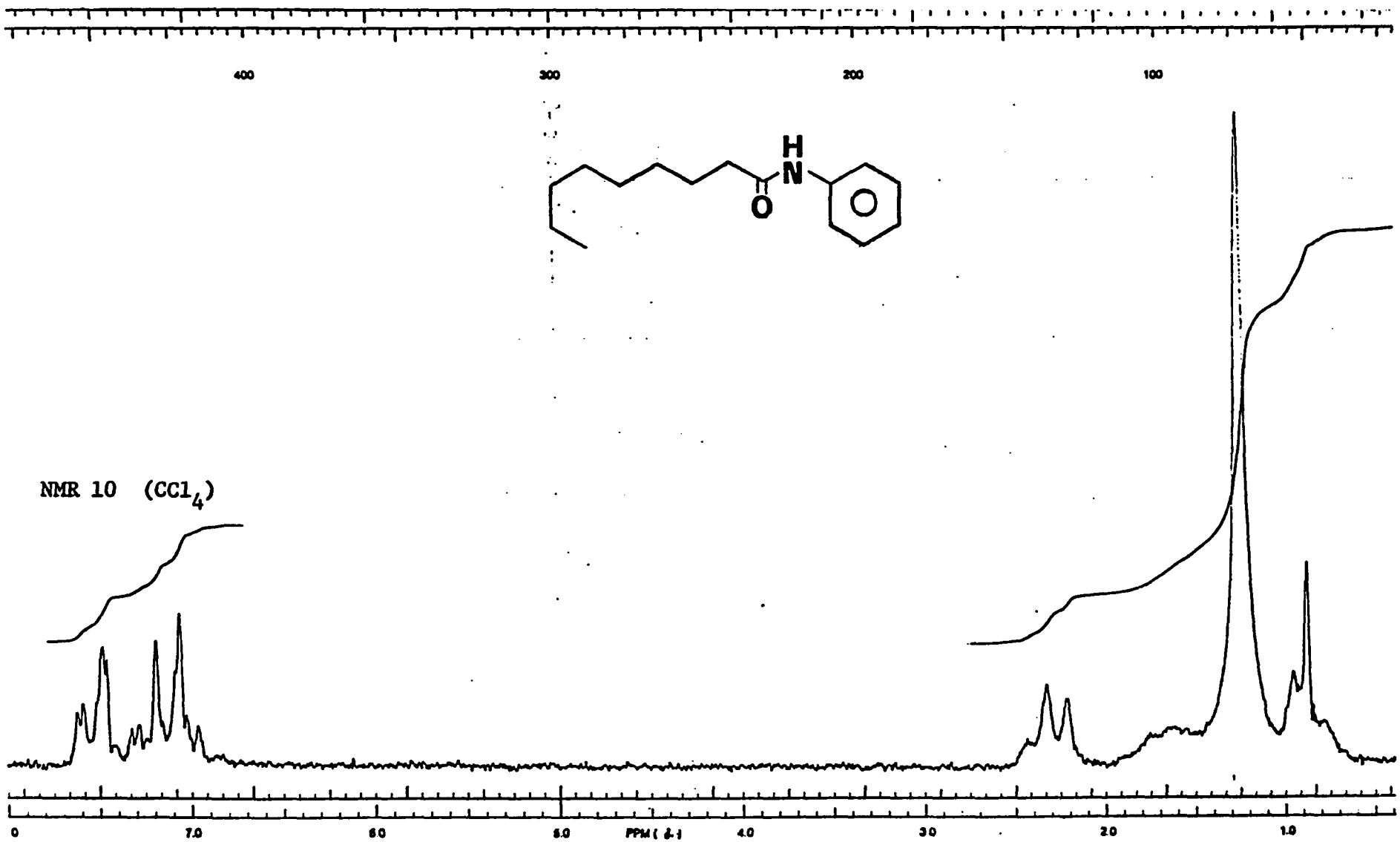
NMR 7 ($CCl_4$)



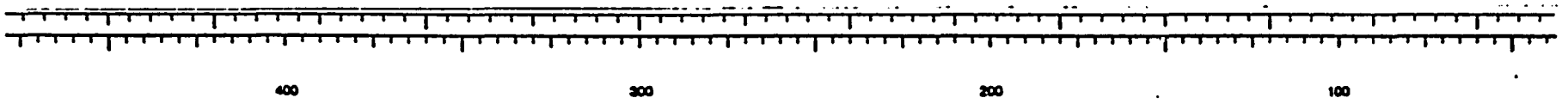

400
300
200
100
S
NH
NMR 8 ($CCl_4$)
8.0
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0

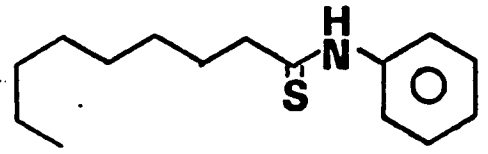



NMR 9 ($CDCl_3$)

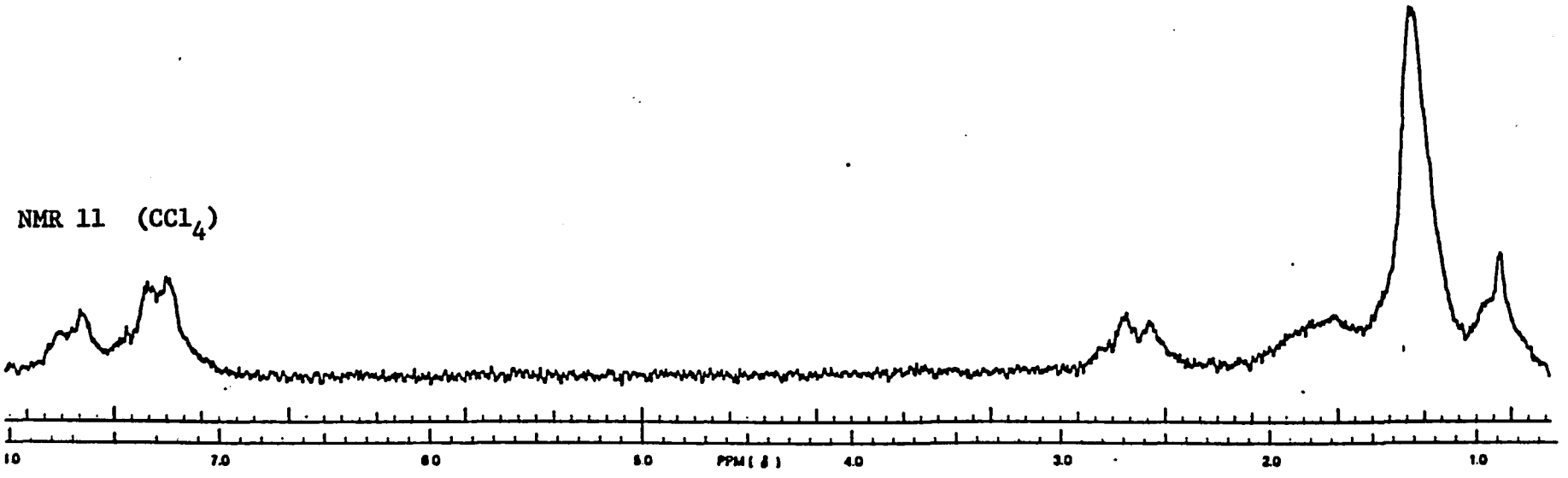


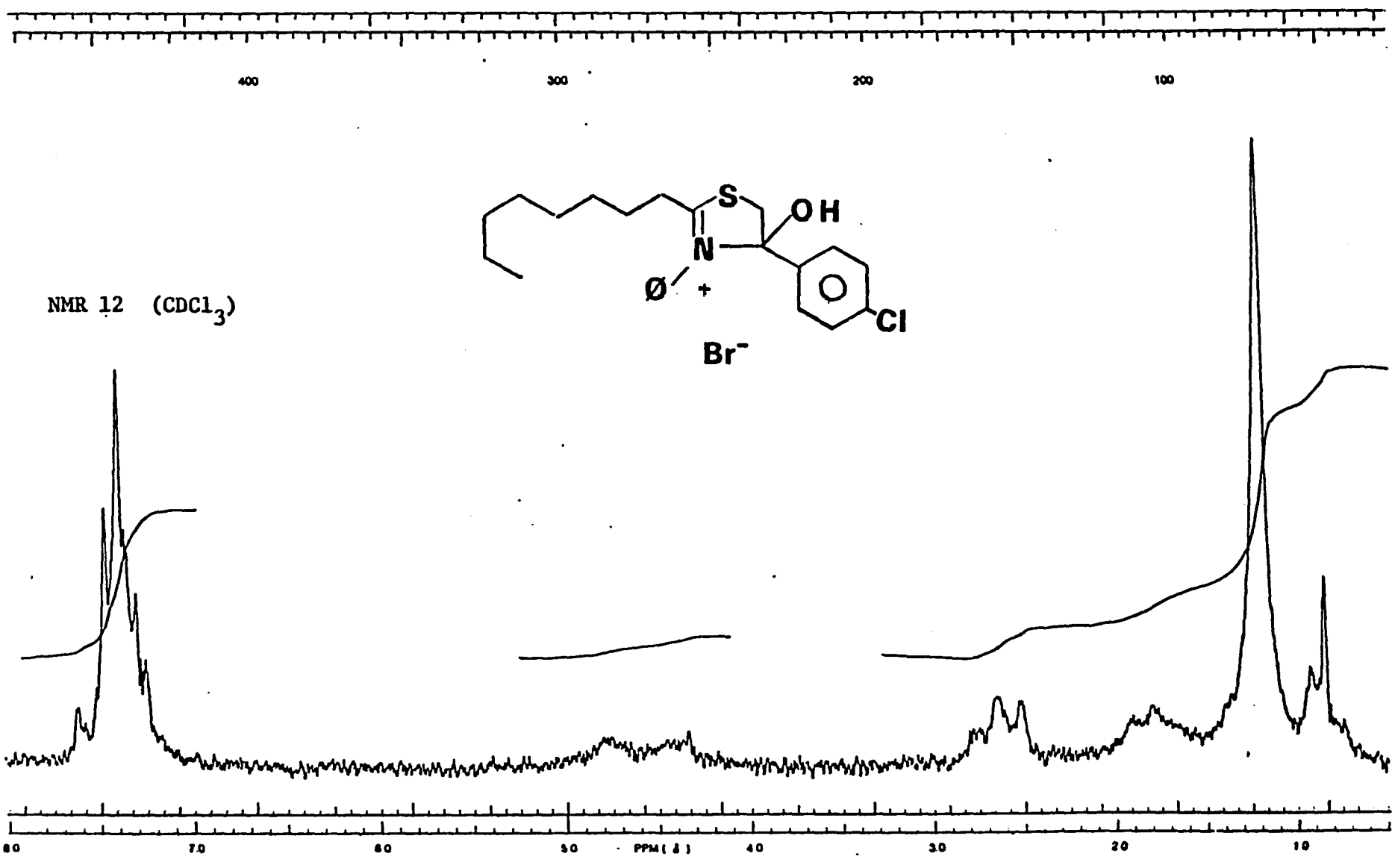

NMR 10 ($CCl_4$)

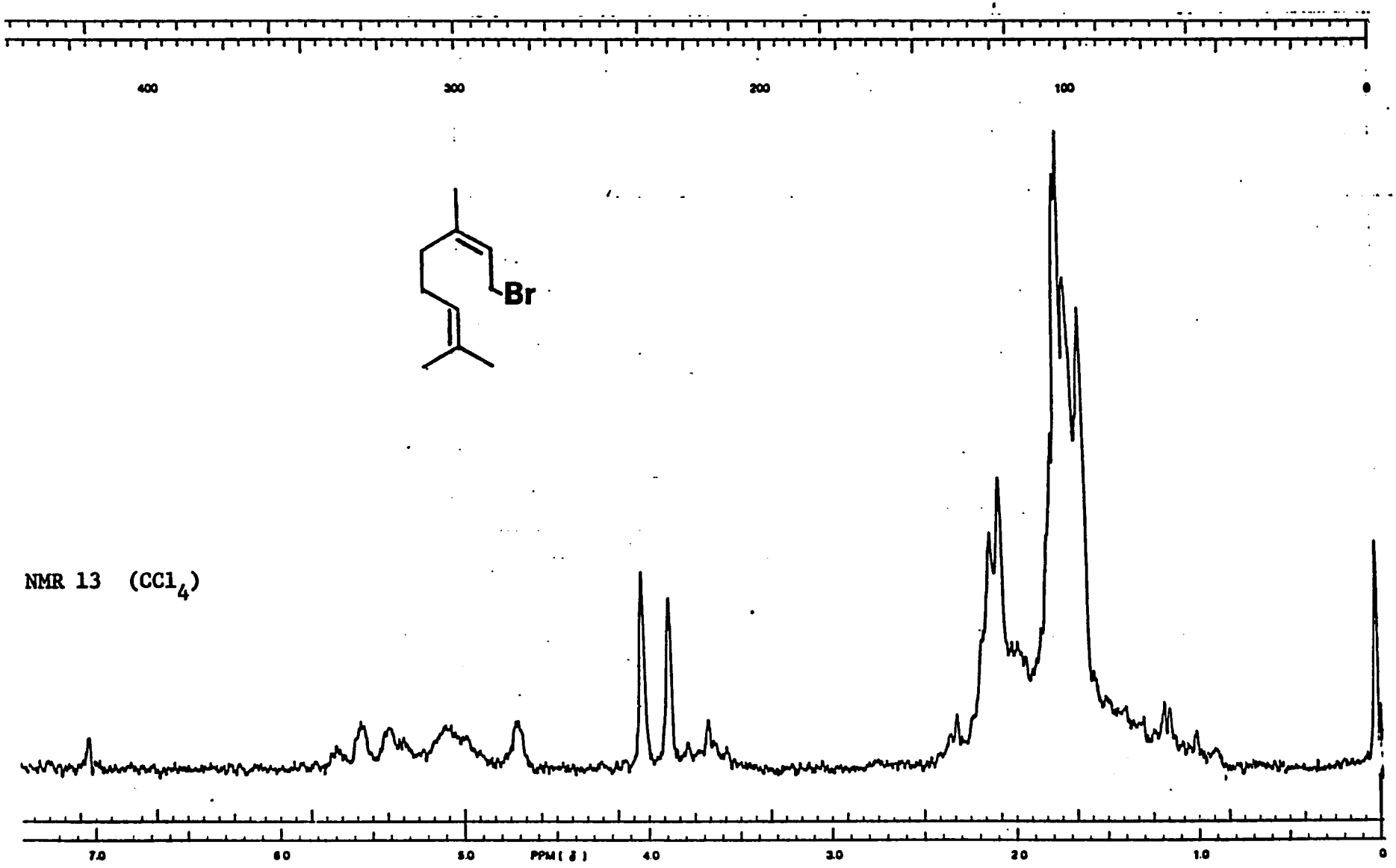


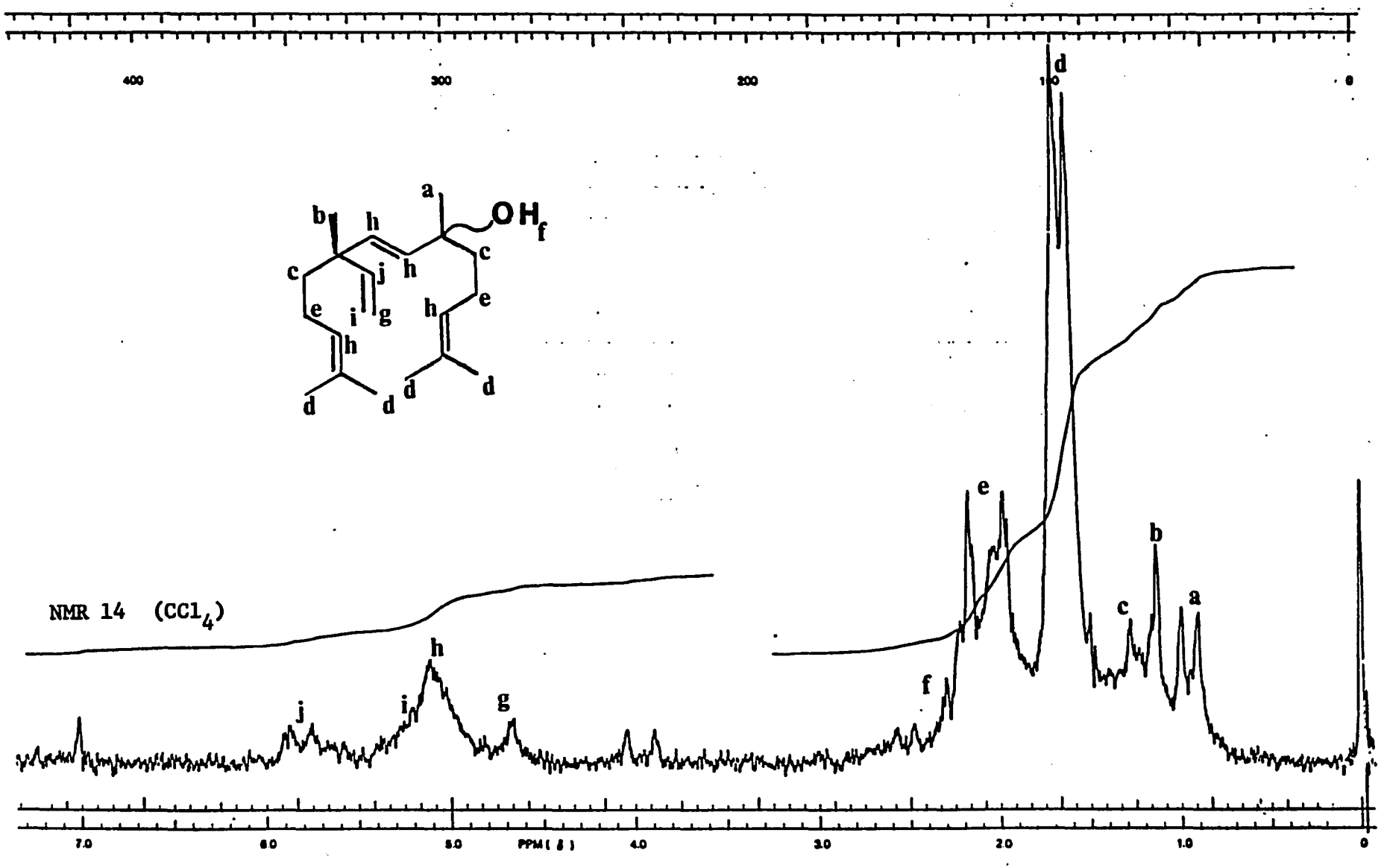



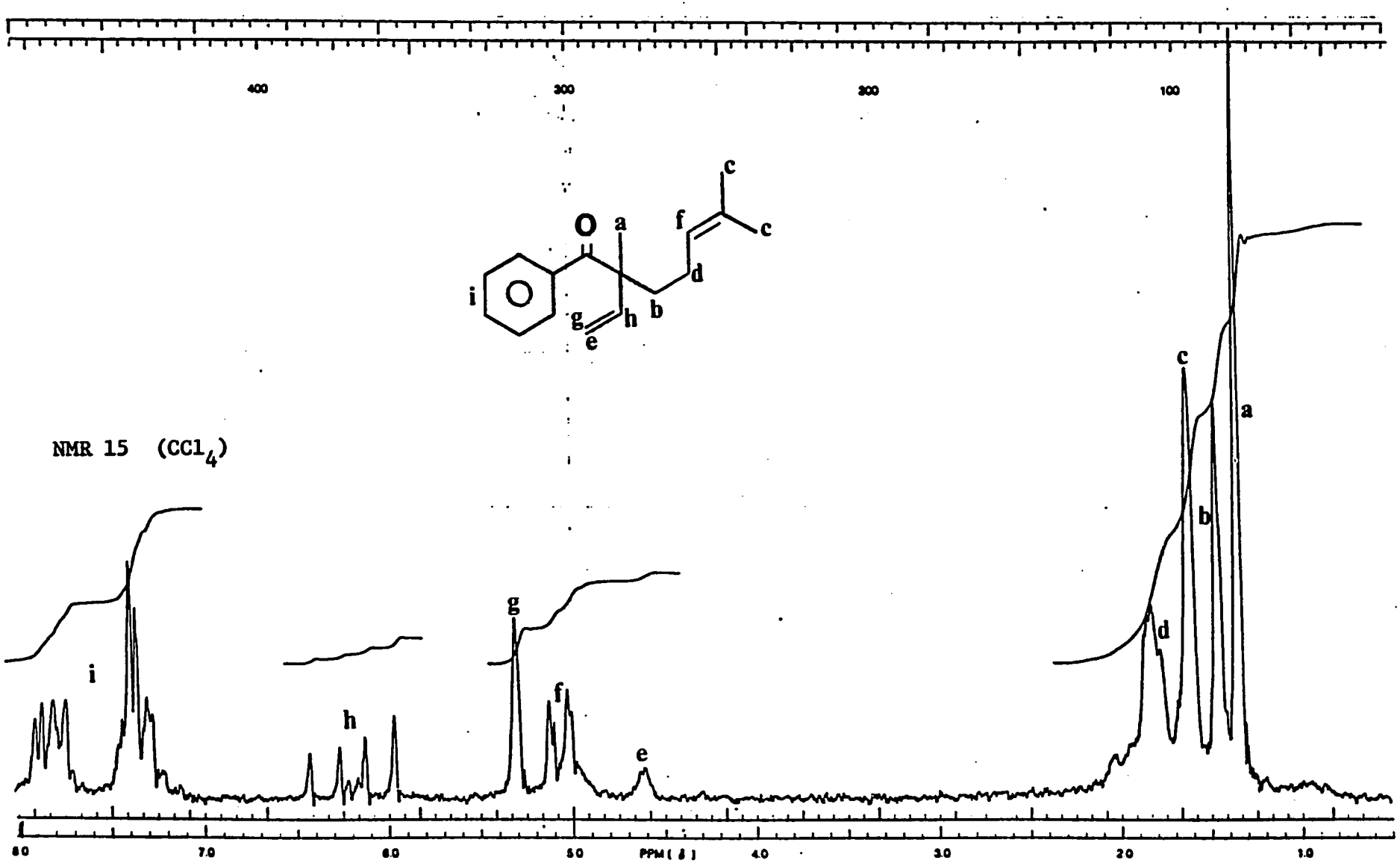

NMR 11 ($CCl_4$)



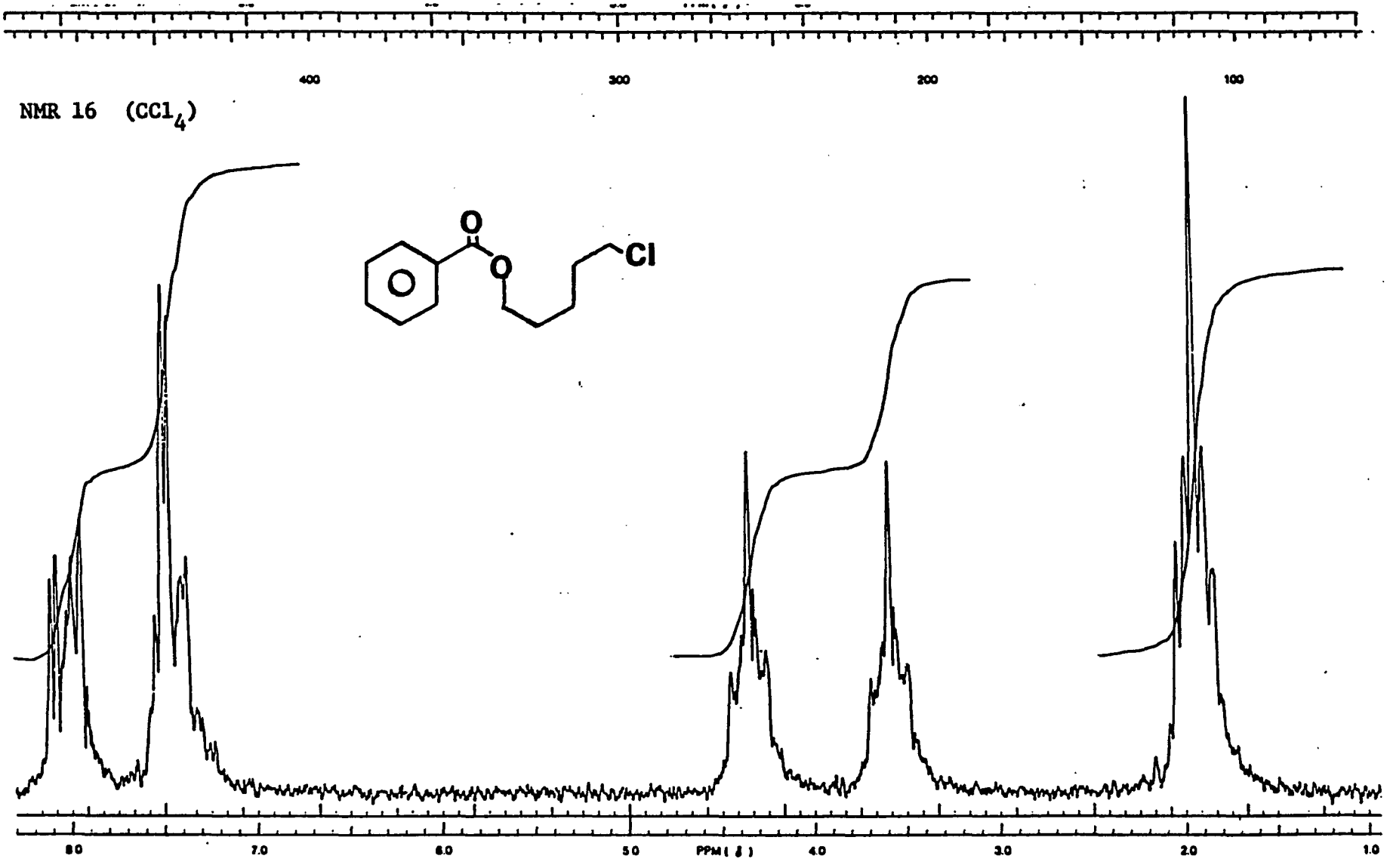

400
300
200
100
S
OH
N
Ø
+
Cl
Br⁻
NMR 12 (CDCl3)
8.0
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0

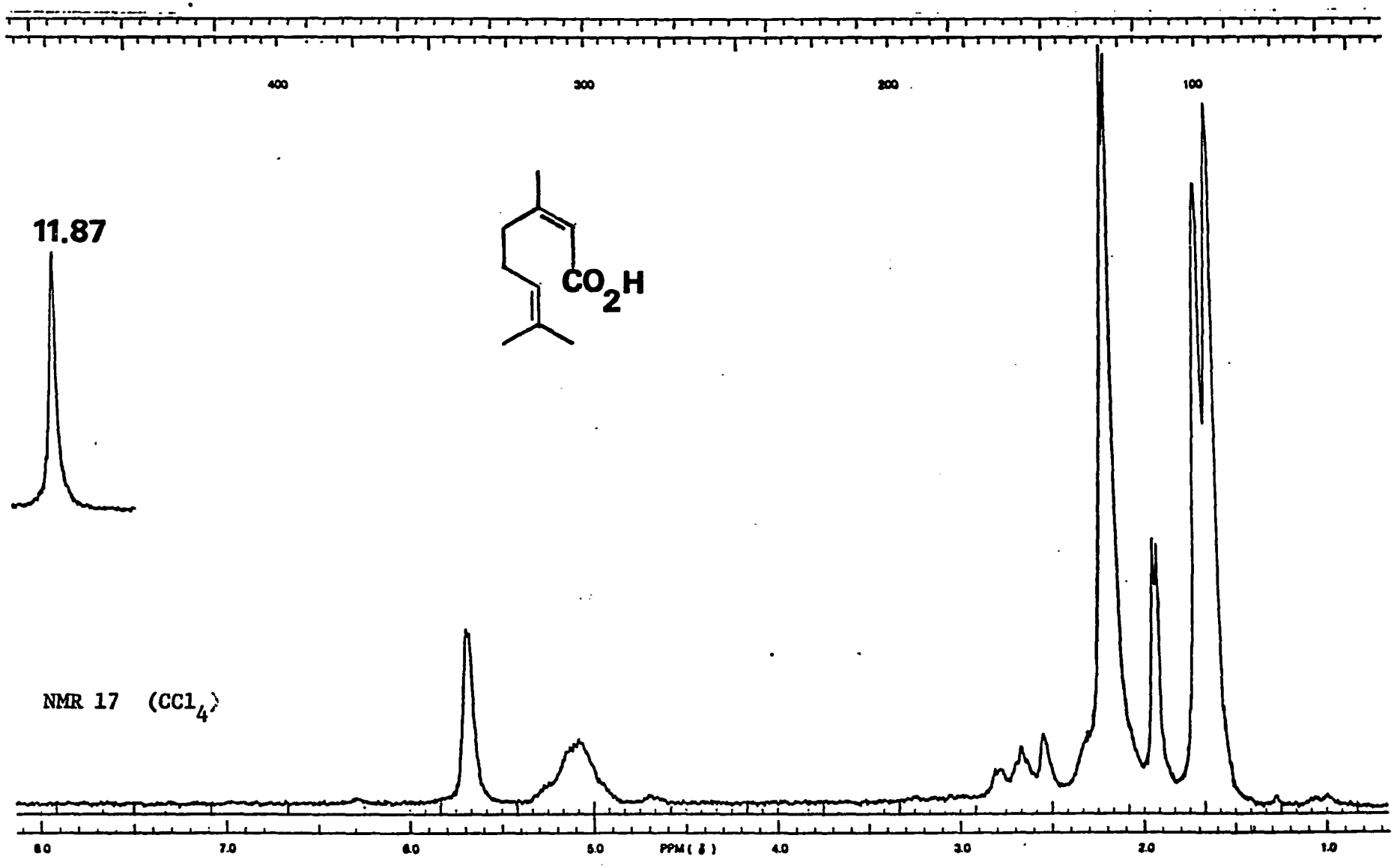

400
300
200
100
0
Br
NMR 13 ($CCl_4$)
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0
0

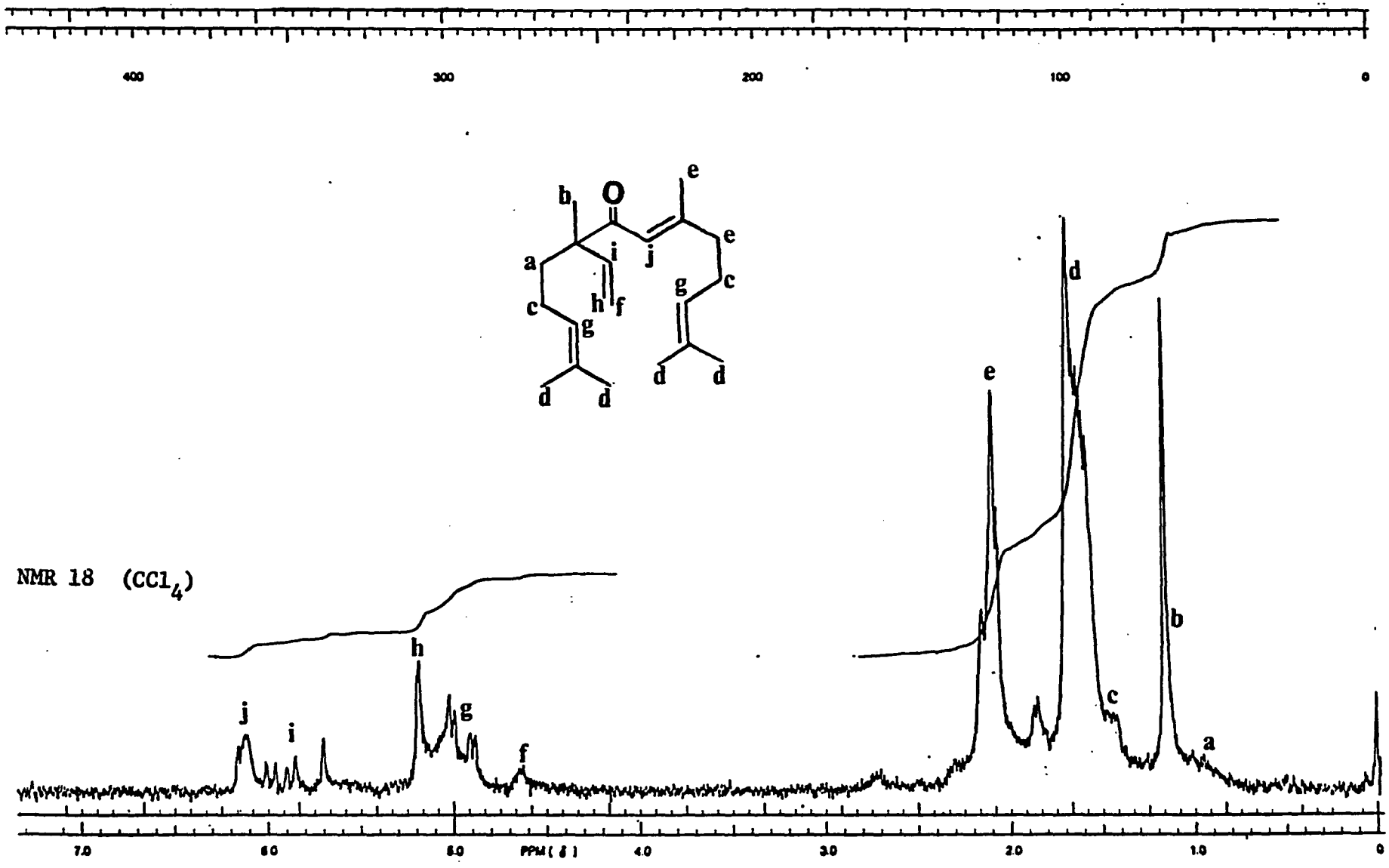

NMR 14 ($CCl_4$)
400
300
200
100
0
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0
0
a
b
c
d
e
f
g
h
i
j
OH
f

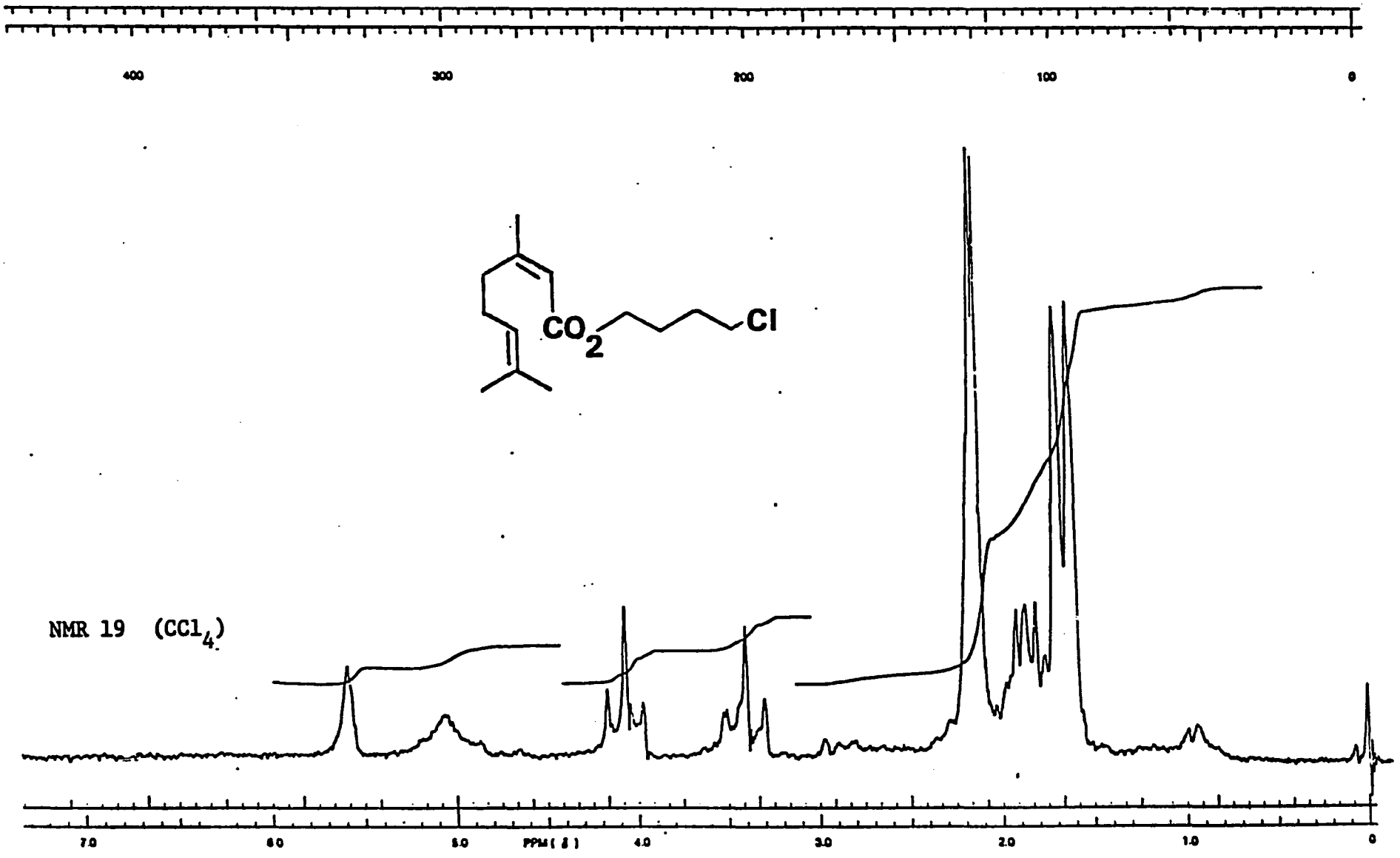

400
300
200
100
O
a
f
c
c
d
i
b
g
h
e
NMR 15 ($CCl_4$)
i
h
g
f
e
d
c
b
a
8.0
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0

NMR 16 ($CCl_4$)

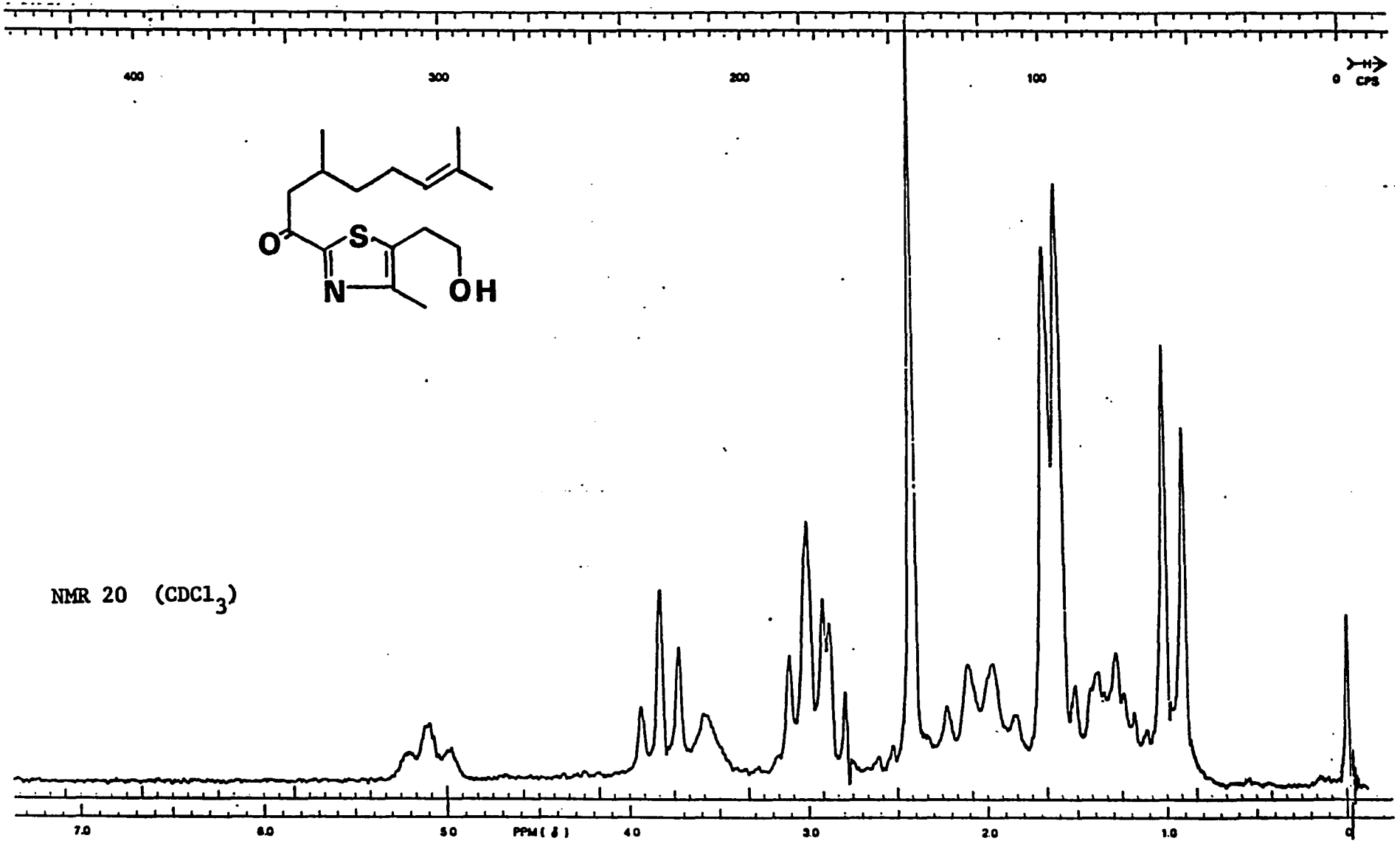


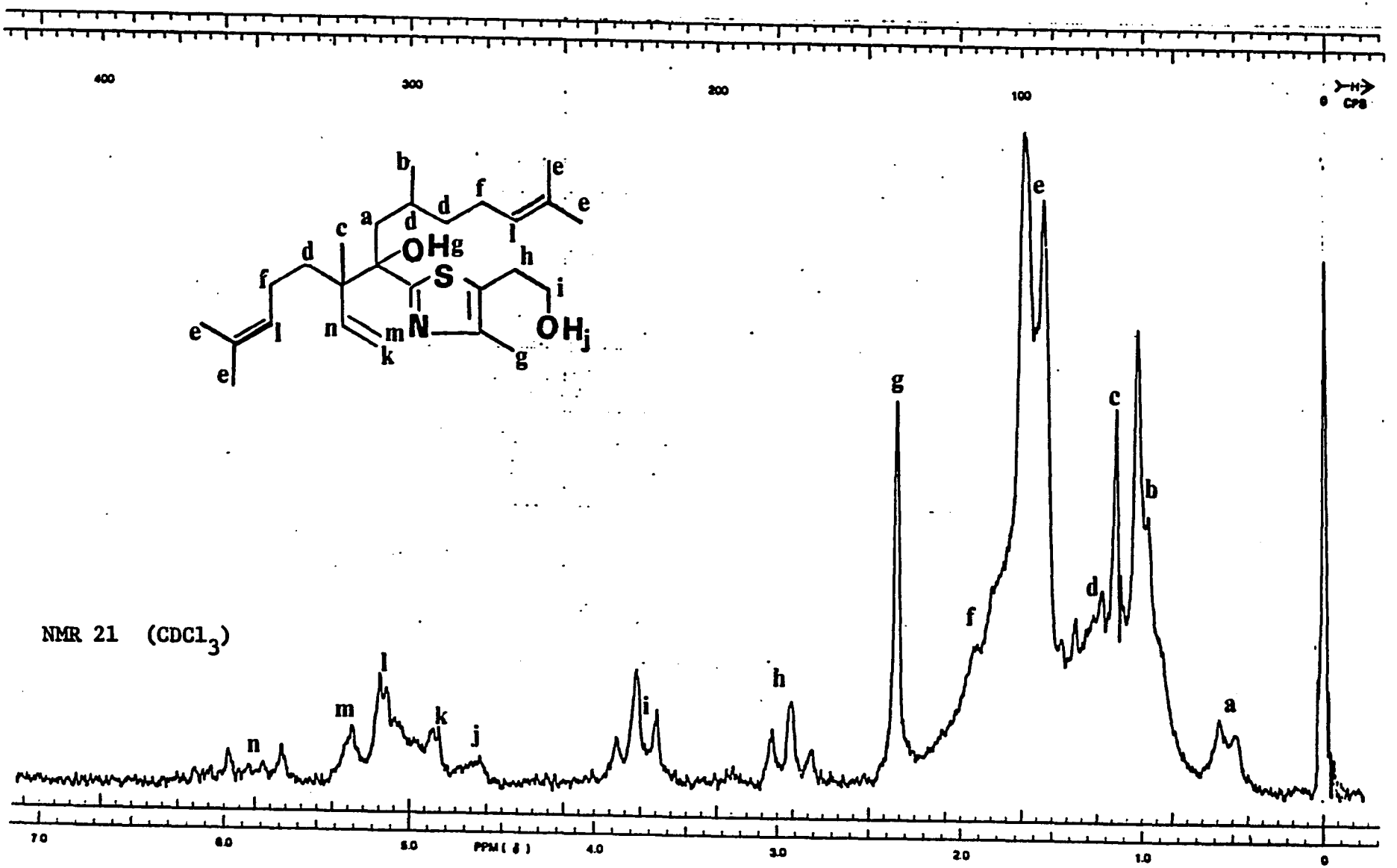

400
300
200
100
11.87
CO2H
NMR 17 (CCl4)
8.0
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0

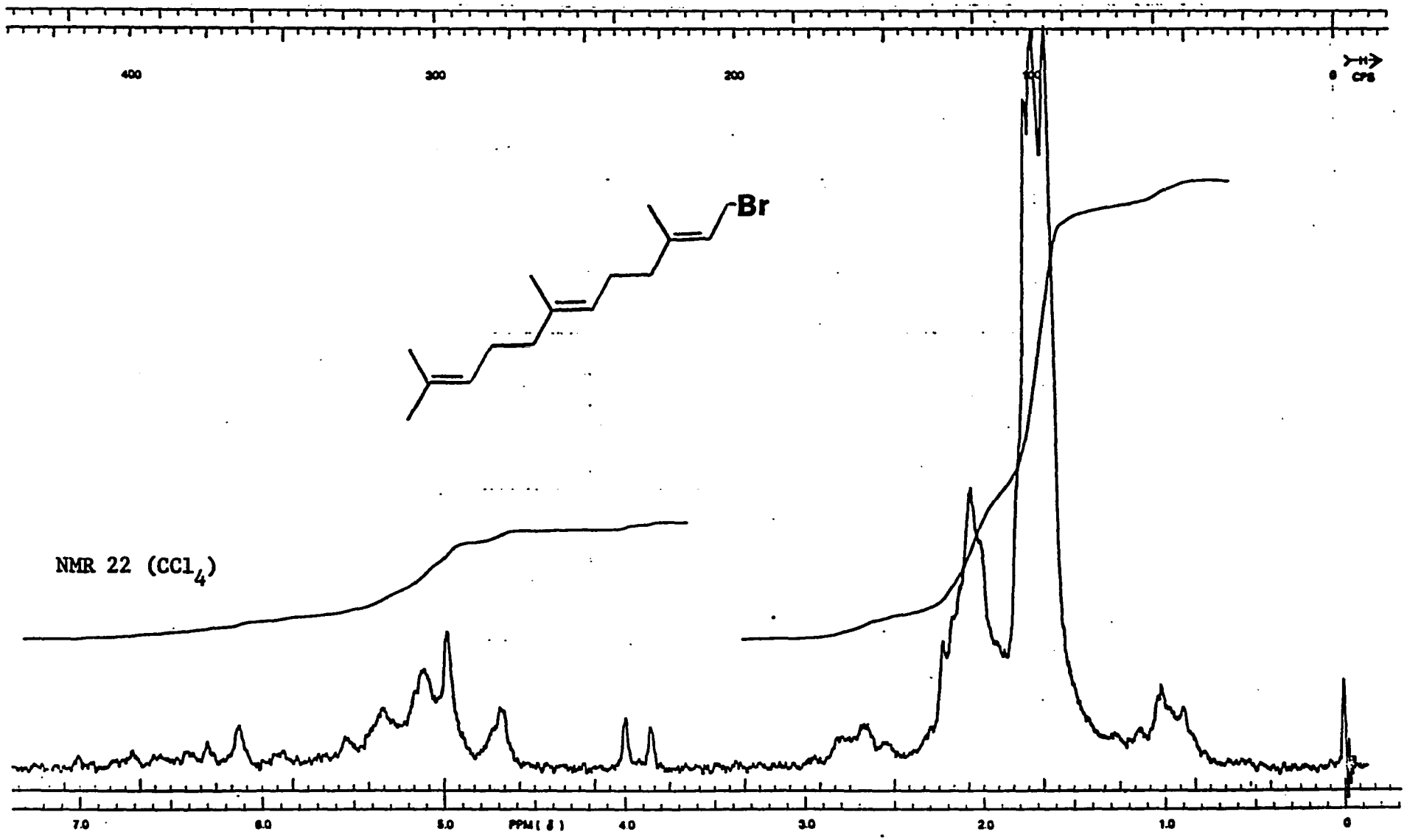

400
300
200
100
0
O
a
b
c
d
e
f
g
h
i
j
NMR 18 ($CCl_4$)
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0
0

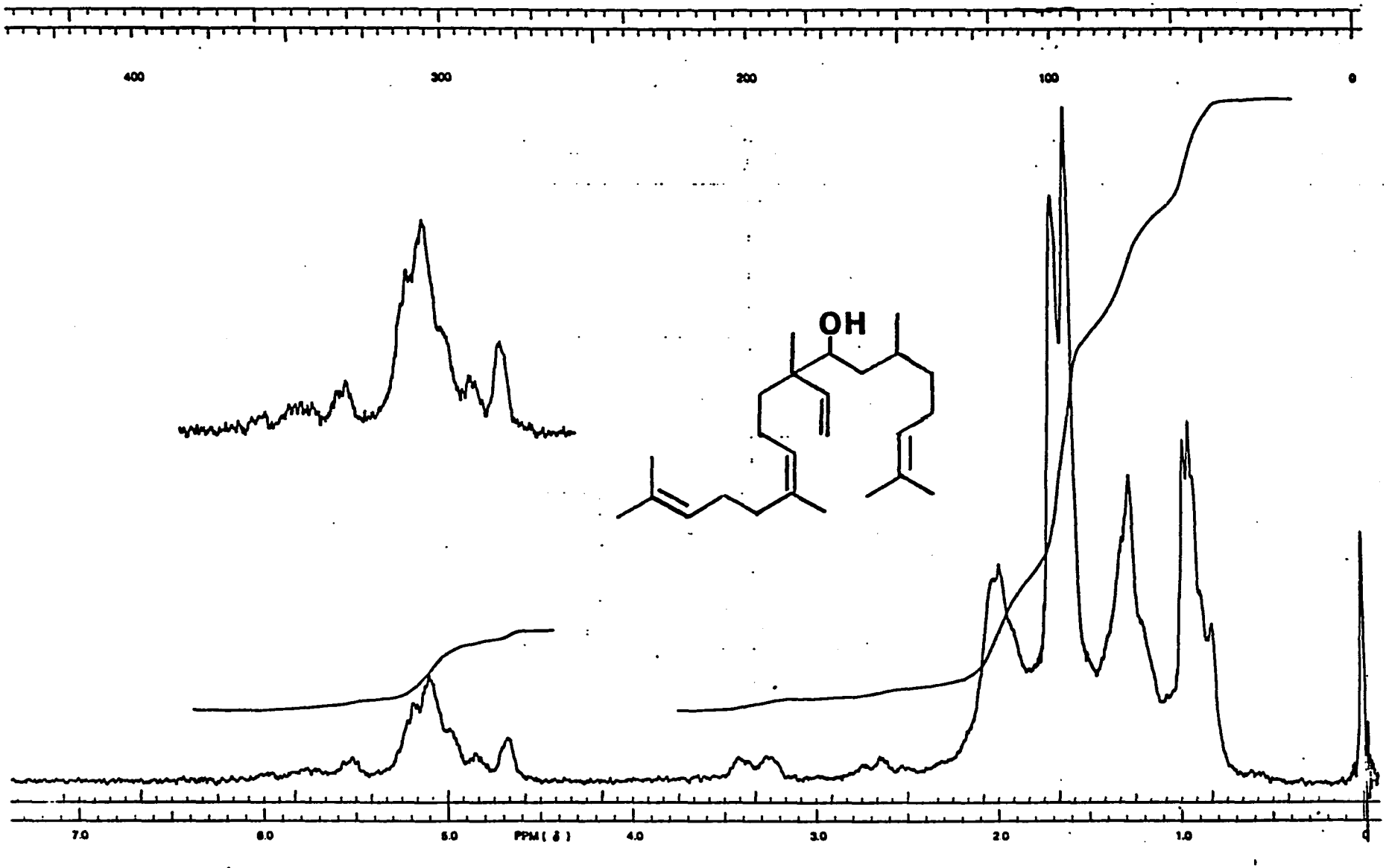



NMR 19 ($CCl_4$)

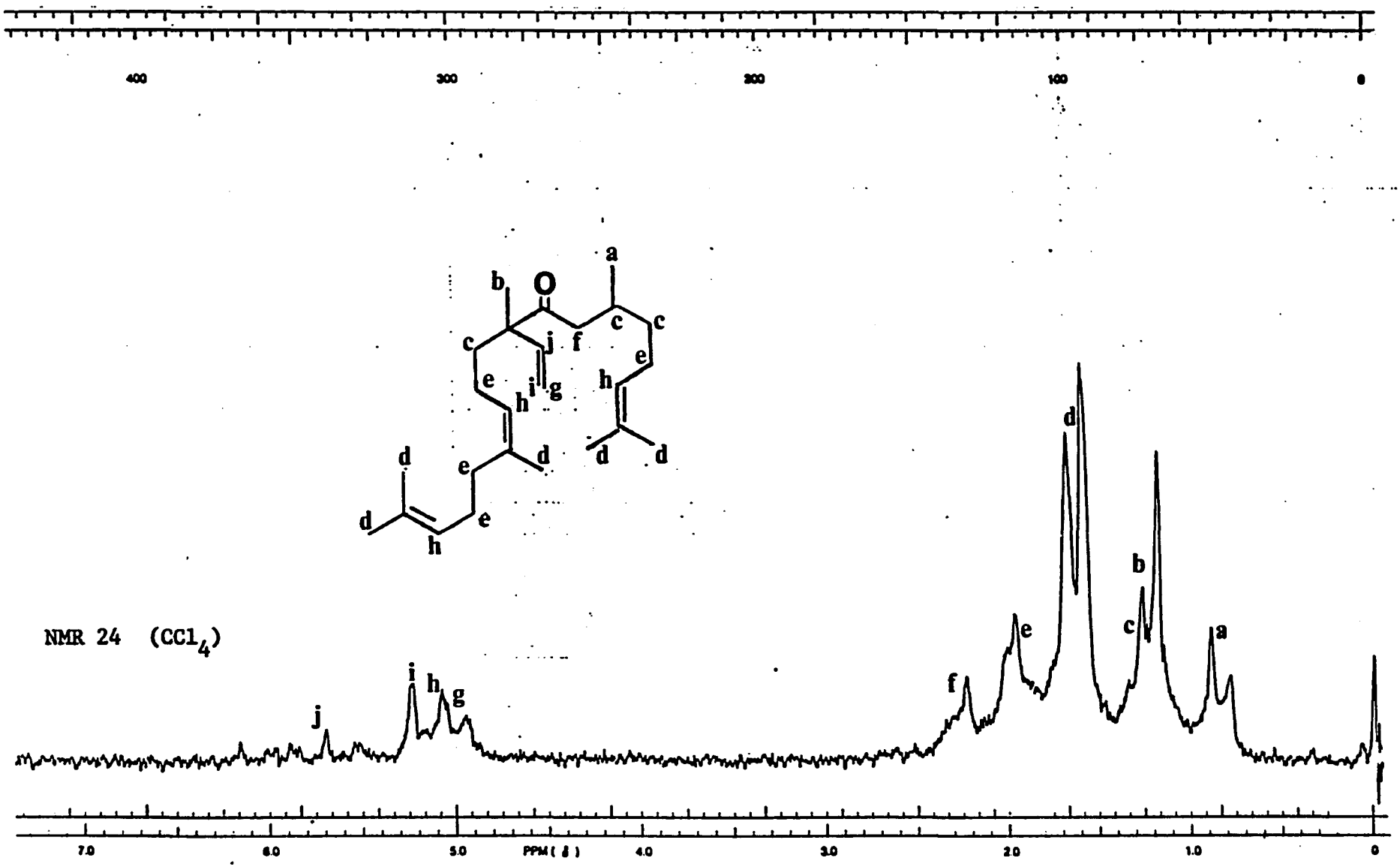

400
300
200
100
0 CPS
O
S
N
OH
NMR 20 (CDCl3)
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0
0

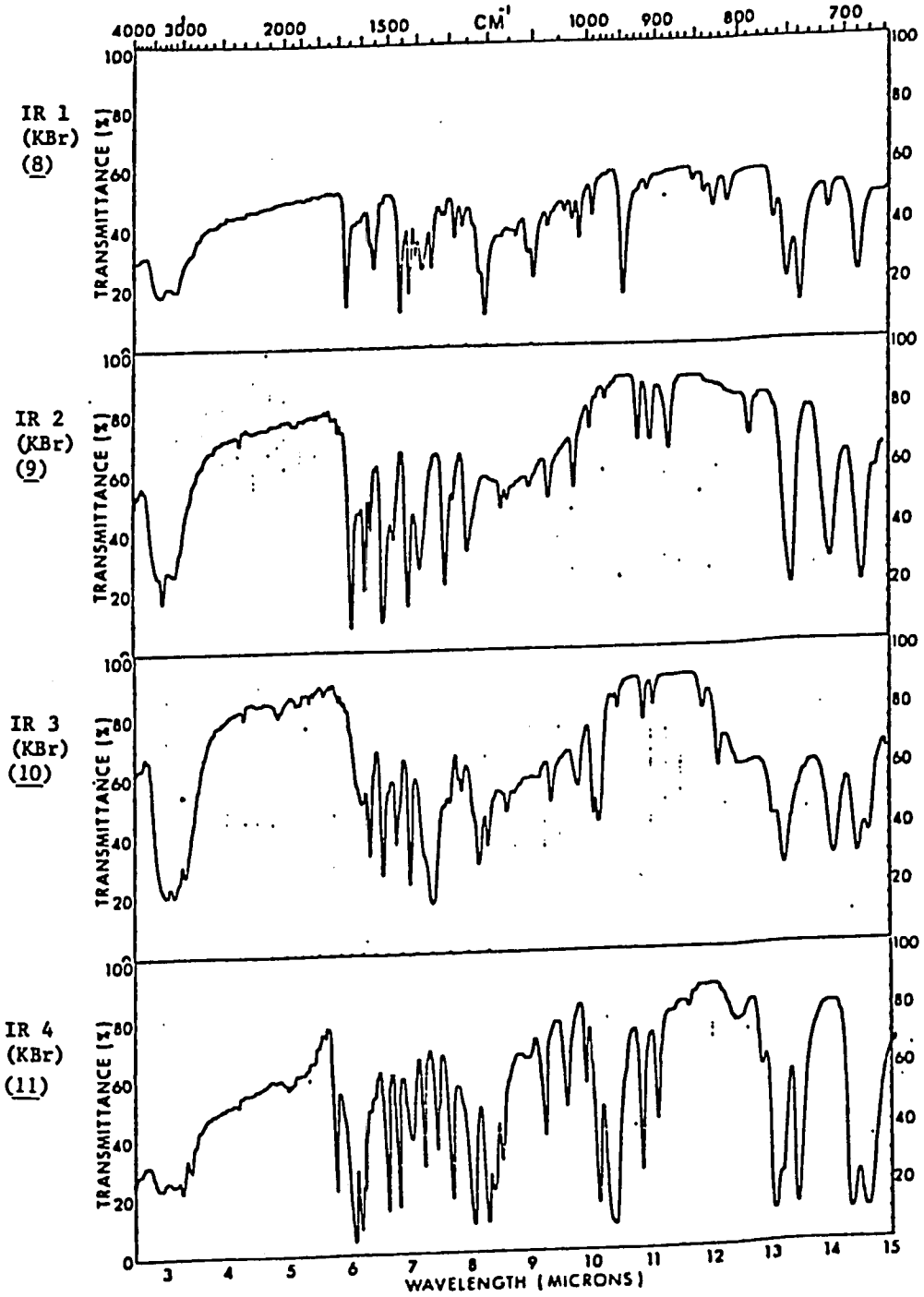

400
300
200
100
0
CPS
NMR 21 (CDCl3)
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0
0

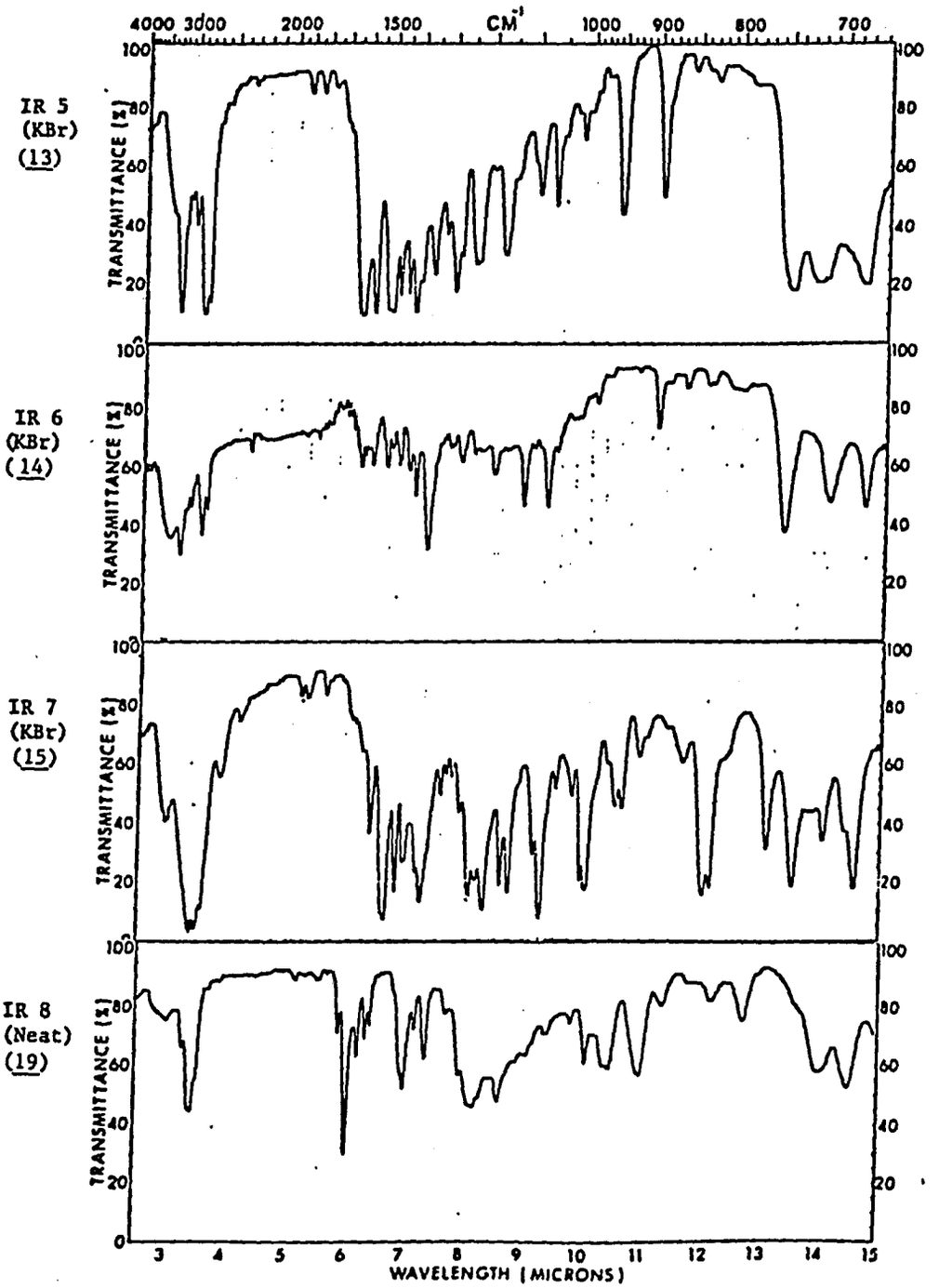

400
300
200
100
0
CPS
Br
NMR 22 ($CCl_4$)
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0
0

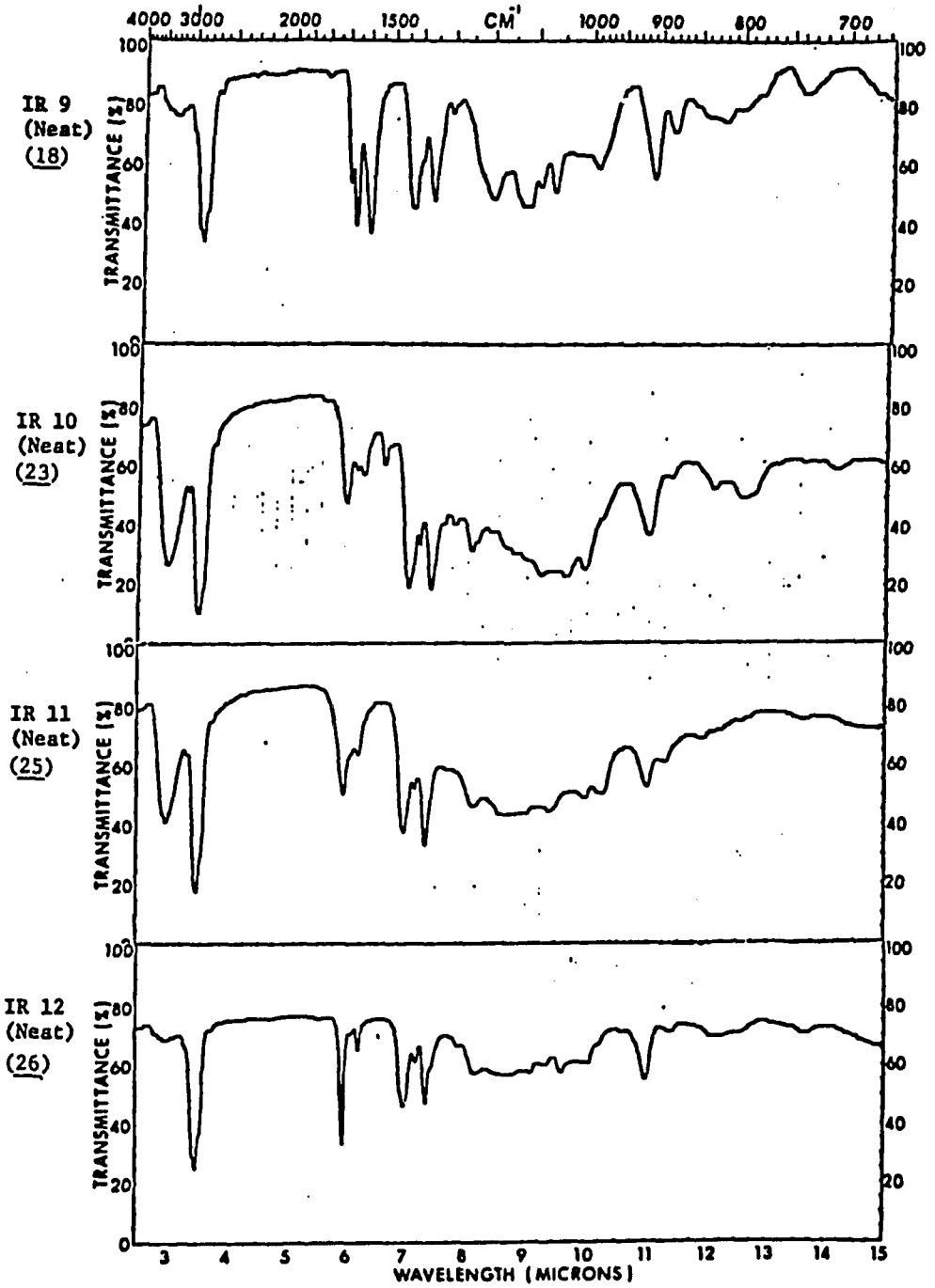


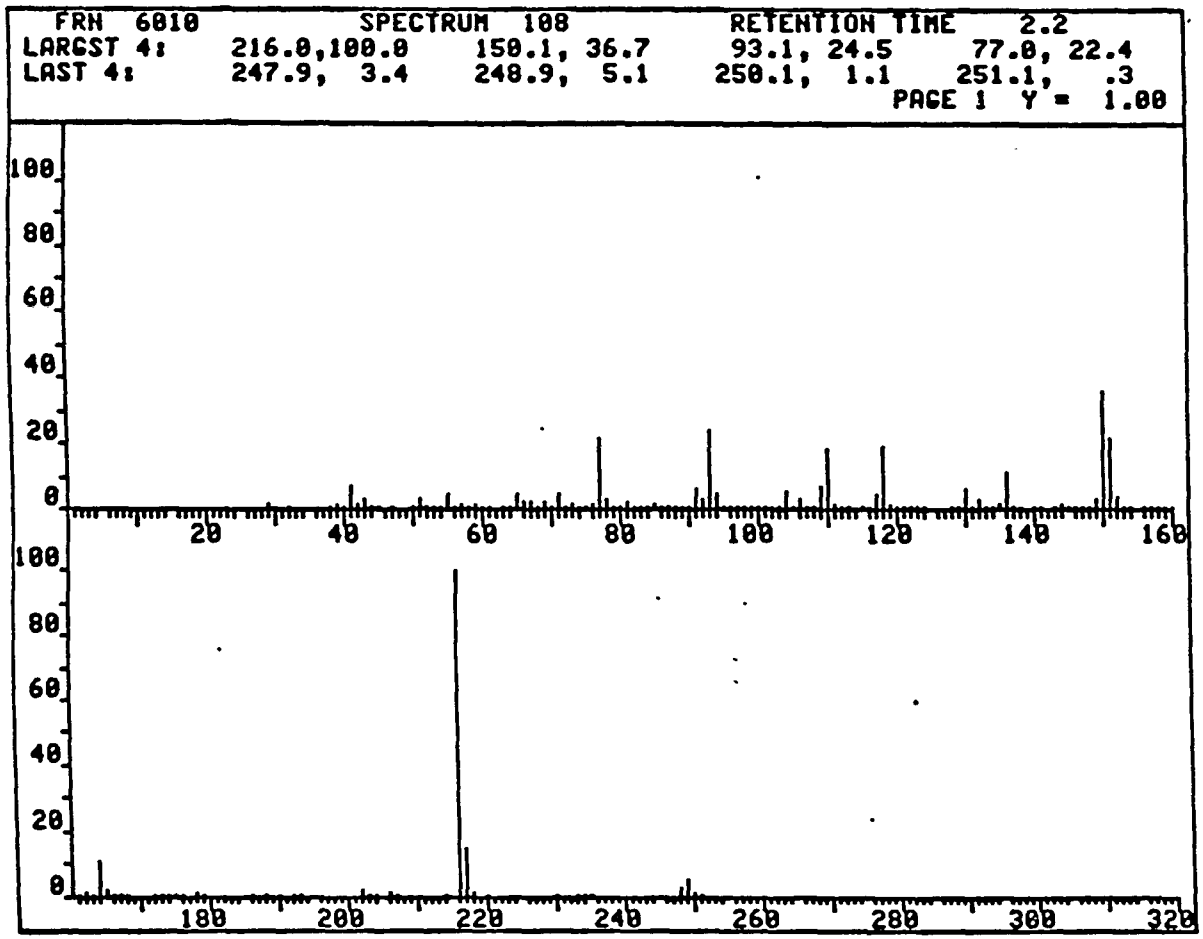

NMR 23 ($CCl_4$)


400
300
200
100
0
a
b
O
c
c
c
j
f
e
e
h
i
g
h
d
d
e
d
d
d
d
e
h
NMR 24 (CCl4)
j
i
h
g
f
e
d
c
b
a
7.0
6.0
5.0
PPM ( δ )
4.0
3.0
2.0
1.0
0


4000 3000 2000 1500 CM⁻¹ 1000 900 800 700
IR 1 (KBr) (8)
IR 2 (KBr) (9)
IR 3 (KBr) (10)
IR 4 (KBr) (11)
TRANSMITTANCE (%)
WAVELENGTH (MICRONS)
3 4 5 6 7 8 9 10 11 12 13 14 15


4000 3000 2000 1500 CM⁻¹ 1000 900 800 700
IR 5 (KBr) (13)
IR 6 (KBr) (14)
IR 7 (KBr) (15)
IR 8 (Neat) (19)
TRANSMITTANCE (%)
100
80
60
40
20
0
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)


4000 3000 2000 1500 CM⁻¹ 1000 900 800 700
IR 9
(Neat)
(18)
IR 10
(Neat)
(23)
IR 11
(Neat)
(25)
IR 12
(Neat)
(26)
TRANSMITTANCE (%)
100
80
60
40
20
0
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)




Mass spectrum #1
(14)

[MS 6010 SPECTRUM 108 REL. TIME = 2.2

>PAUSE

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 29 | 1.9 | 64 | .7 | 90 | .7 | 117 | 5.3 |
| 32 | 1.3 | 65 | 5.2 | 91 | 6.8 | | |
| | | 66 | 2.3 | 92 | 3.9 | 118 | 19.2 |
| 38 | .2 | 67 | 2.5 | 93 | 24.5 | 119 | 1.9 |
| 39 | 1.9 | 68 | .5 | 94 | 4.9 | 120 | 1.2 |
| 40 | .8 | 69 | 2.3 | 95 | .7 | 121 | .3 |
| 41 | 7.2 | 70 | .5 | 96 | .2 | 122 | .2 |
| 42 | 1.7 | 71 | 5.2 | 97 | .6 | 123 | .4 |
| 43 | 3.4 | 72 | .6 | 98 | .2 | 124 | .3 |
| 44 | .5 | 73 | 1.3 | 99 | .4 | 128 | .5 |
| 45 | 1.2 | 74 | .4 | 100 | .2 | 129 | .2 |
| 47 | .3 | 75 | .9 | 101 | .2 | 130 | 6.6 |
| | | | | 102 | .2 | 131 | 1.3 |
| 50 | .7 | 76 | 1.7 | 103 | .9 | | |
| 51 | 2.9 | 77 | 22.4 | | | 132 | 3.1 |
| 52 | .6 | 78 | 3.3 | 104 | 5.5 | 133 | .6 |
| 53 | .9 | 79 | 1.2 | 105 | 1.0 | 134 | .5 |
| 54 | .8 | 80 | .3 | 106 | 3.3 | 135 | 1.4 |
| 55 | 5.4 | 81 | 2.6 | 107 | .3 | 136 | 11.1 |
| 56 | .9 | 82 | .7 | 108 | .8 | 137 | 1.1 |
| 57 | 1.9 | 83 | .4 | 109 | 7.1 | 138 | .6 |
| 58 | 1.1 | 84 | .4 | 110 | 18.5 | 140 | .2 |
| 59 | 1.6 | 85 | 1.8 | 111 | 2.0 | 141 | .2 |
| 60 | .8 | 86 | .2 | 112 | 1.0 | 143 | .8 |
| 61 | .3 | 87 | .5 | 113 | .2 | 144 | 1.6 |
| | | 88 | .2 | 115 | .6 | 145 | .5 |
| 63 | .6 | 89 | .8 | 116 | .7 | | |

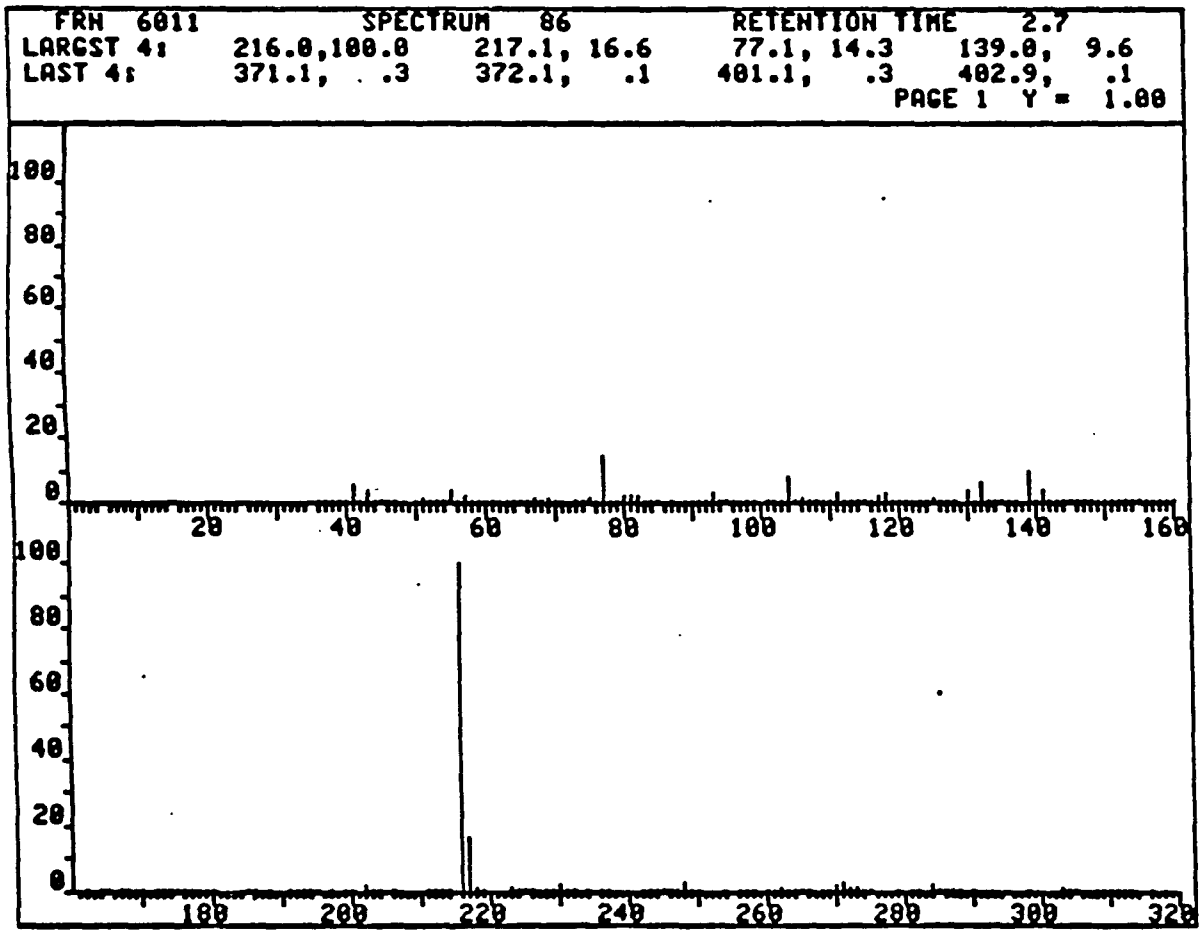
<CONT>

MS #1

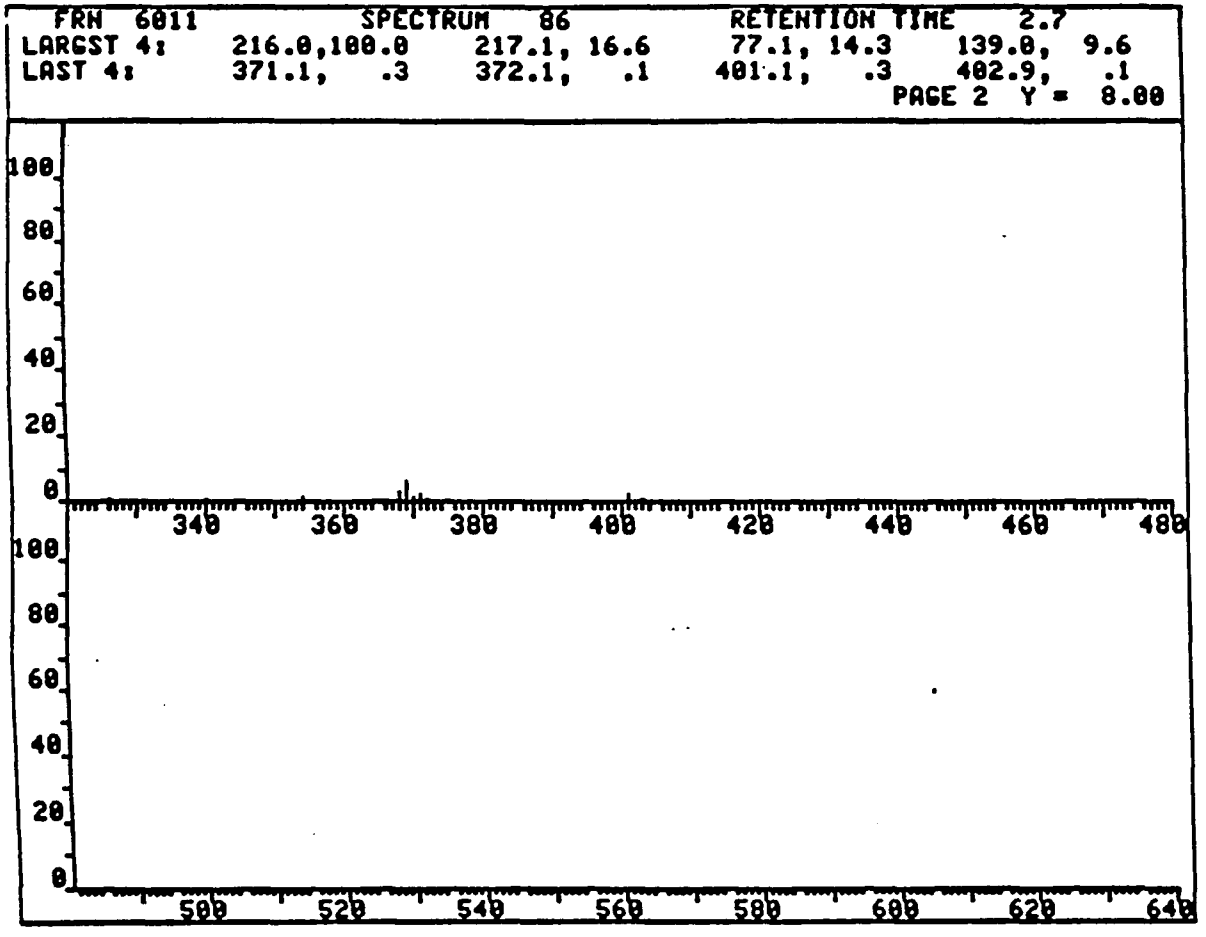
FKN 6010 SPECTRUM 100 REL. TIME = 2.2

| MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|
| 146 | .9 | 175 | .2 | 248 | 3.4 |
| 147 | .3 | 176 | .4 | 249 | 5.1 |
| 148 | 1.0 | 178 | 1.5 | 250 | 1.1 |
| 149 | 3.6 | 179 | .3 | 251 | .3 |
| 150 | 36.7 | 186 | .2 | >PAUSE | |
| 151 | 21.4 | | | | |
| 152 | 4.4 | 188 | .2 | | |
| 153 | 1.1 | 190 | .2 | | |
| 154 | .2 | 192 | .8 | | |
| 156 | .4 | 193 | .2 | | |
| 157 | .2 | | | | |
| 158 | 1.3 | 202 | 2.0 | | |
| 159 | .4 | 203 | .3 | | |
| | | 204 | .3 | | |
| 160 | .7 | 206 | 1.0 | | |
| 161 | .2 | 207 | .2 | | |
| 162 | 1.4 | 214 | .2 | | |
| 163 | .3 | | | | |
| 164 | 11.1 | 216 | 100.0 | | |
| 165 | 2.3 | 217 | 14.9 | | |
| 166 | .6 | 218 | 1.4 | | |
| 167 | .3 | 220 | .5 | | |
| 168 | .2 | | | | |
| 172 | .5 | 230 | .3 | | |
| 173 | .4 | 233 | .4 | | |
| | | 234 | .3 | | |
| 174 | .4 | 235 | .2 | | |

MS #1




Mass spectrum #2
(15)


FRN 6011 SPECTRUM 86 RETENTION TIME 2.7
LARGST 4: 216.0,100.0 217.1, 16.6 77.1, 14.3 139.0, 9.6
LAST 4: 371.1, .3 372.1, .1 401.1, .3 402.9, .1
PAGE 2 Y = 8.00
100
80
60
40
20
0
340
360
380
400
420
440
460
480
100
80
60
40
20
0
500
520
540
560
580
600
620
640

MS #2

FBN 6011 SPECTRUM 86 RET. TIME = 2.7
>PAUSE

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 36 | .1 | 63 | .3 | 91 | 1.0 | 118 | 3.6 |
| 37 | .1 | 64 | .4 | 92 | .6 | 119 | .4 |
| 38 | .2 | 65 | 1.1 | 93 | 3.3 | 120 | .9 |
| 39 | 1.2 | 66 | .7 | 94 | .7 | 121 | .3 |
| 40 | .5 | 67 | 1.4 | 95 | .4 | 122 | .1 |
| 41 | 6.1 | 68 | .3 | 96 | .2 | 123 | .2 |
| 42 | .6 | 69 | 1.7 | 97 | .3 | 124 | .1 |
| 43 | 4.5 | 70 | .2 | 98 | .1 | 125 | 1.4 |
| 44 | .5 | 71 | .8 | 99 | .2 | 126 | .2 |
| 45 | .6 | 73 | .4 | 101 | .2 | 127 | .5 |
| 46 | .1 | 74 | .4 | 102 | .1 | 128 | .3 |
| 47 | .1 | 75 | 1.7 | 103 | .6 | 130 | 4.1 |
| | | | | | | 131 | .9 |
| 50 | .7 | 76 | 1.3 | 104 | 8.3 | | |
| 51 | 1.9 | 77 | 14.3 | 105 | .8 | 132 | 6.7 |
| 52 | .3 | 78 | 1.1 | 106 | 1.4 | 133 | .8 |
| 53 | .5 | 79 | 1.2 | 107 | .3 | 134 | .2 |
| 54 | .2 | 80 | 2.2 | 108 | .2 | 135 | .4 |
| 55 | 4.4 | 81 | 2.6 | 109 | .8 | 136 | .8 |
| 56 | .3 | 82 | 2.1 | 110 | .3 | 137 | .3 |
| 57 | 2.1 | 83 | .3 | 111 | 3.1 | 139 | 9.6 |
| 58 | .2 | 84 | .2 | 112 | .5 | 140 | .9 |
| 59 | .1 | 85 | .5 | 113 | 1.2 | 141 | 4.8 |
| 60 | .1 | 87 | .2 | 114 | .2 | 142 | .3 |
| 61 | .2 | 89 | .7 | 115 | .2 | 143 | .4 |
| | | | | 116 | .2 | 144 | 1.2 |
| 62 | .1 | 90 | .4 | 117 | 2.2 | 145 | .3 |
| | | | | | | <CONT> | |

MS #2

FRM 6011 SPECTRUM 86 RET. TIME = 2.7
<PAUSE>

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 146 | .5 | 175 | .1 | 216 | 100.0 | 254 | .1 |
| 147 | .1 | 176 | .1 | 217 | 16.6 | 256 | .8 |
| 148 | .3 | 177 | .1 | 218 | 1.3 | 257 | .2 |
| 149 | .9 | 178 | .1 | 219 | .1 | | |
| 150 | .8 | 179 | .1 | 223 | 1.6 | 258 | .4 |
| 151 | .2 | 180 | .1 | 224 | .3 | 260 | .1 |
| 152 | .2 | 185 | .1 | 225 | .7 | 261 | .1 |
| 153 | .3 | 186 | .2 | 226 | .1 | 262 | 1.3 |
| 154 | .3 | | | 228 | .2 | 263 | .2 |
| 155 | .2 | 188 | .2 | 229 | .1 | 264 | .2 |
| 156 | .2 | 191 | .1 | | | 265 | .2 |
| 157 | .2 | 192 | .2 | 230 | 1.7 | 266 | .1 |
| 158 | .7 | 193 | .1 | 231 | .3 | 268 | .2 |
| 159 | .5 | 194 | .1 | 232 | .1 | 269 | .1 |
| | | 195 | .1 | 234 | .1 | 270 | 2.4 |
| 160 | .4 | 197 | .1 | 236 | .1 | 271 | 2.9 |
| 162 | .2 | 199 | .1 | 237 | .1 | | |
| 164 | .5 | 200 | .2 | 242 | .7 | 272 | 1.5 |
| 165 | .2 | | | 243 | .6 | 273 | 1.2 |
| 167 | .2 | 202 | 1.9 | | | 274 | .2 |
| 168 | .3 | 203 | .3 | 244 | .5 | 275 | .1 |
| 169 | .3 | 204 | .2 | 245 | .3 | 279 | .1 |
| 170 | .2 | 206 | .1 | 246 | .1 | 281 | .1 |
| 172 | .8 | 211 | .2 | 247 | .1 | 282 | .1 |
| 173 | .3 | 212 | .1 | 248 | 2.7 | 284 | 1.9 |
| | | 213 | .2 | 249 | .5 | 285 | .4 |
| 174 | .1 | 214 | .7 | 250 | .2 | | |

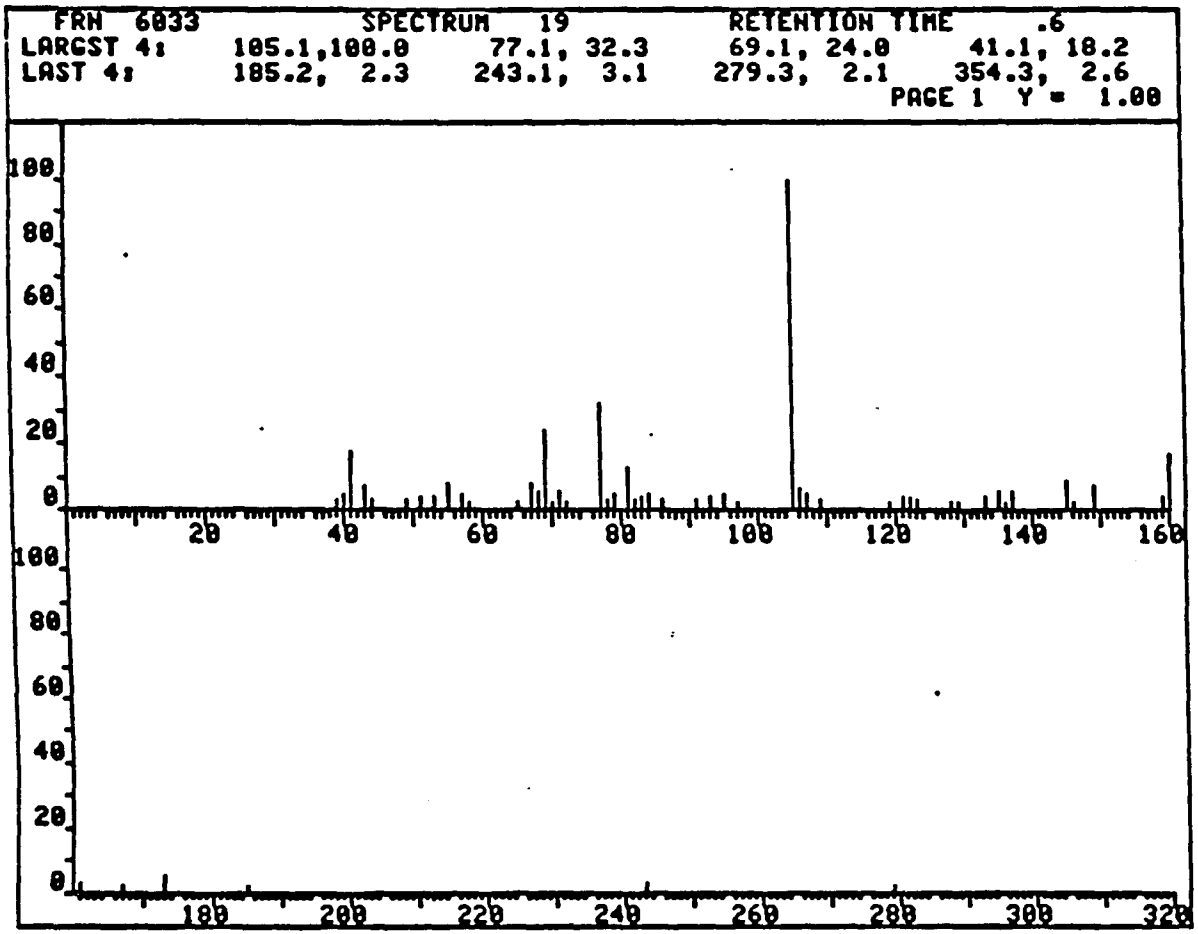
<CONT>

MS #2

FRN 6011 SPECTRUM 86 RET. TIME = 2.7

| MASS | ABUND | MASS | ABUND |
|---|---|---|---|
| 286 | .6 | 370 | .2 |
| 287 | .1 | 371 | .3 |
| 288 | .2 | 372 | .1 |
| 290 | .1 | | |
| 298 | .1 | 401 | .3 |
| | | 403 | .1 |
| 303 | 1.2 | >PAUSE | |
| 304 | .3 | | |
| 305 | .5 | | |
| 308 | .4 | | |
| 309 | .6 | | |
| 310 | .3 | | |
| 311 | .3 | | |
| 312 | .1 | | |
| 313 | .1 | | |
| 316 | .2 | | |
| 318 | .1 | | |
| 326 | .1 | | |
| 340 | .1 | | |
| 354 | .2 | | |
| 368 | .4 | | |
| 369 | .8 | | |

MS #2

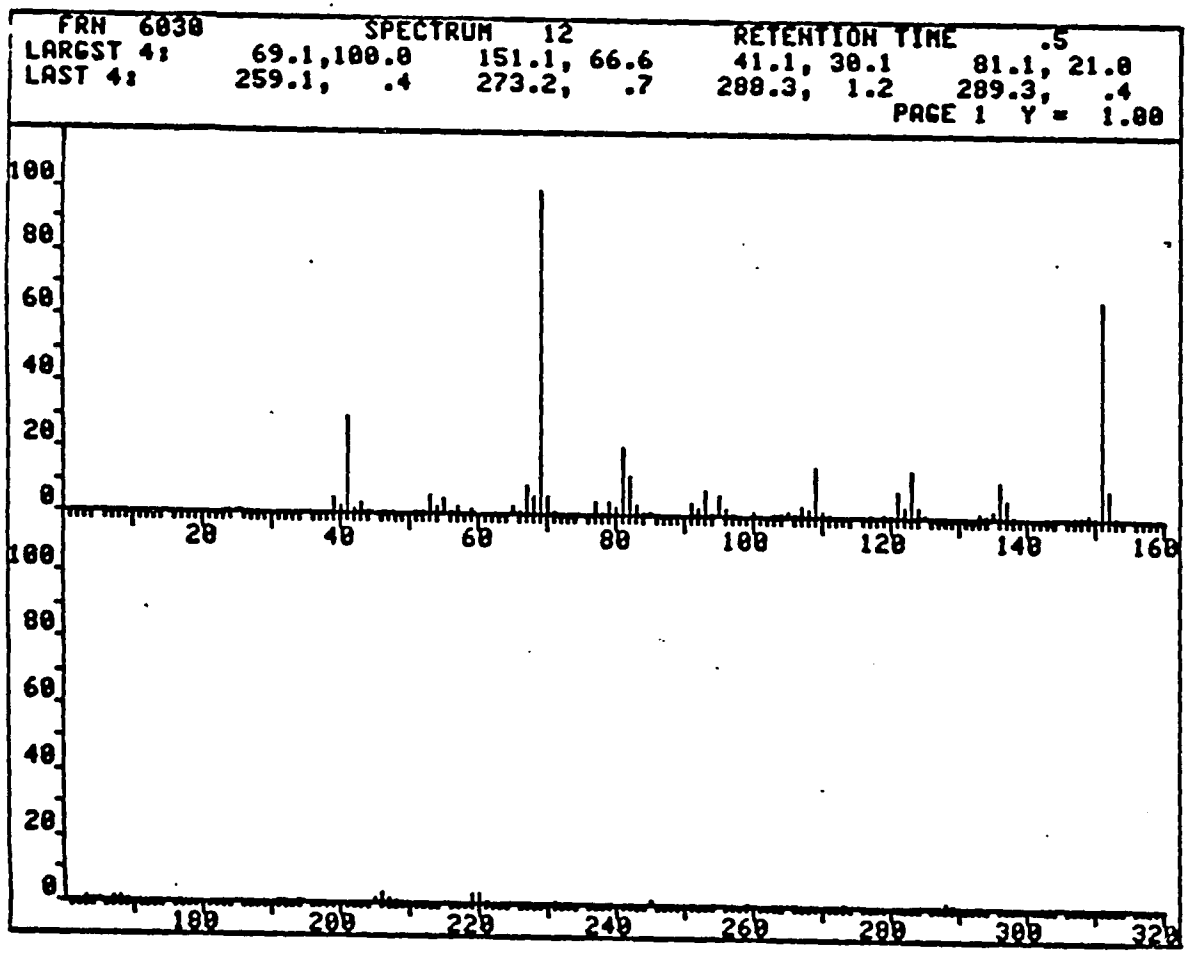



Mass spectrum #3
(19)

FRM 6033 SPECTRUM 19 RET. TIME = .6

| MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|
| 39 | 3.6 | 84 | 5.2 | 149 | 7.6 |
| 40 | 4.9 | 86 | 3.1 | 159 | 4.2 |
| 41 | 18.2 | | | | |
| 43 | 7.3 | 91 | 3.6 | 160 | 17.2 |
| 44 | 3.4 | 93 | 4.4 | 161 | 3.4 |
| | | 95 | 4.7 | 167 | 2.1 |
| 49 | 3.6 | 97 | 2.6 | 173 | 5.5 |
| 51 | 3.9 | | | | |
| 53 | 4.2 | 185 | 100.0 | 185 | 2.3 |
| 55 | 8.3 | 186 | 6.8 | | |
| 57 | 5.2 | 187 | 4.7 | 243 | 3.1 |
| 58 | 2.6 | 189 | 3.1 | | |
| | | | | 279 | 2.1 |
| 65 | 2.3 | 119 | 2.3 | | |
| 67 | 8.3 | 121 | 4.4 | 354 | 2.6 |
| 68 | 6.0 | 122 | 3.9 | >PAUSE | |
| 69 | 24.0 | 123 | 3.6 | | |
| 70 | 2.1 | 128 | 2.3 | | |
| 71 | 6.0 | 129 | 2.1 | | |
| 72 | 2.1 | | | | |
| | | 133 | 3.9 | | |
| 77 | 32.3 | 135 | 5.5 | | |
| 78 | 3.4 | 136 | 2.6 | | |
| 79 | 5.2 | 137 | 6.0 | | |
| 81 | 13.3 | 145 | 9.4 | | |
| 82 | 3.6 | | | | |
| 83 | 3.9 | 146 | 2.3 | | |

MS #3

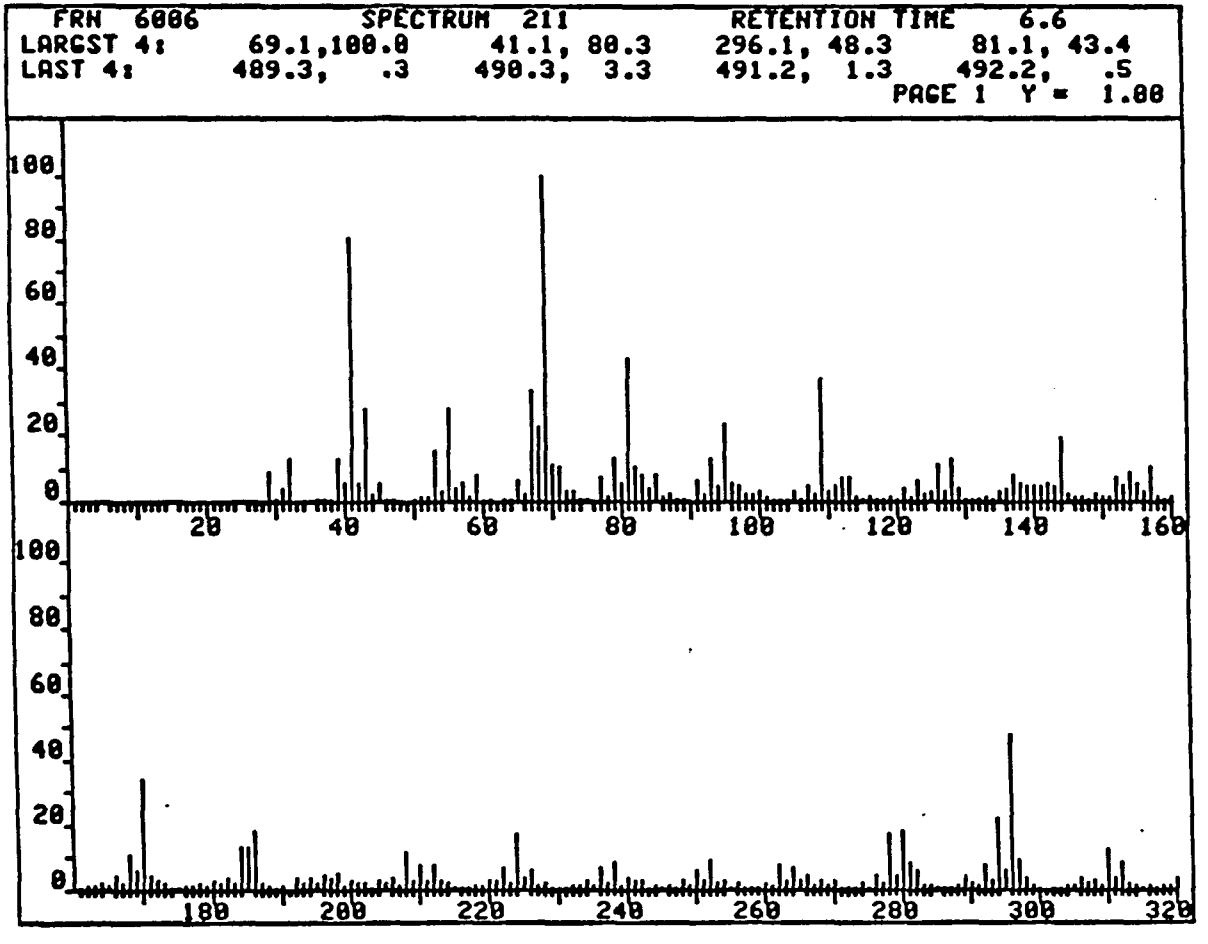



Mass spectrum #4
(18)

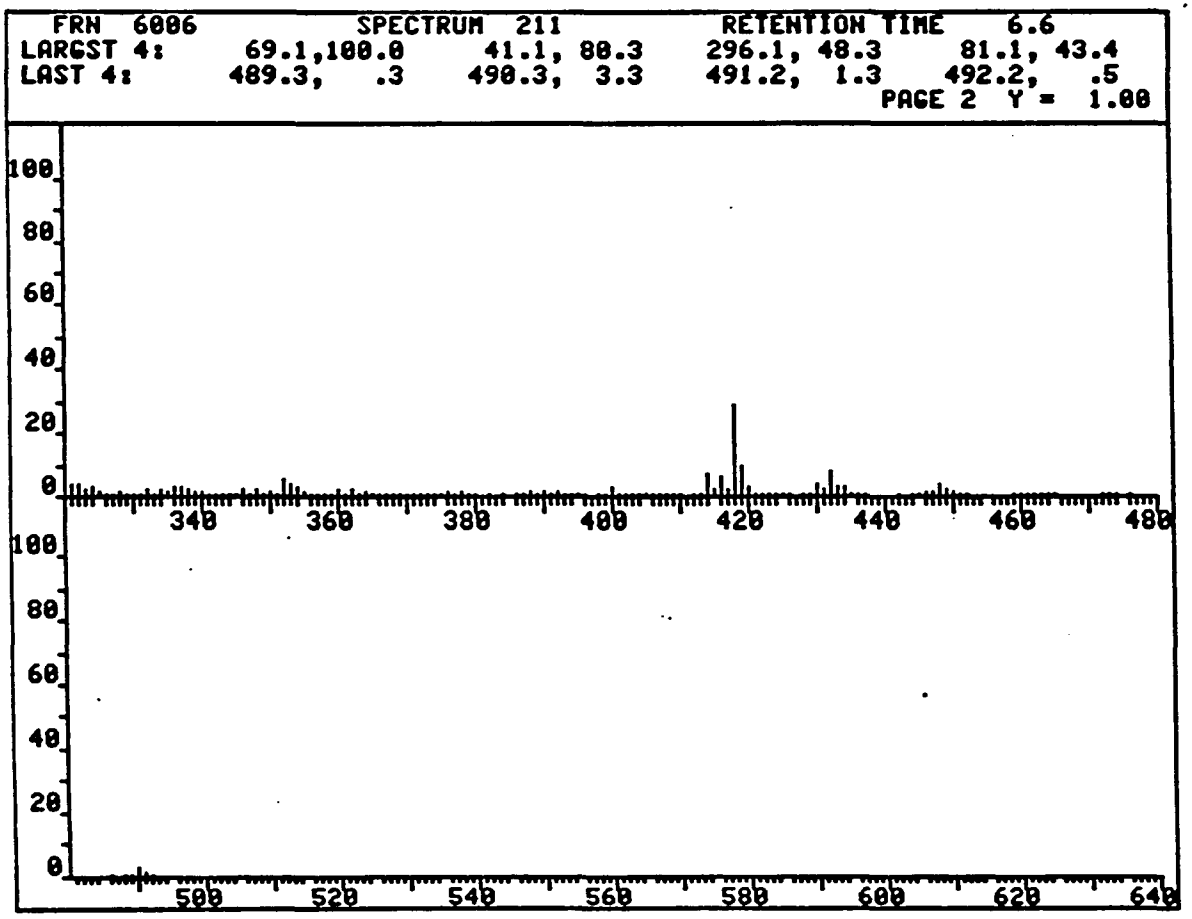
FRH 6030 SPECTRUM 12 RET. TIME = .5

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 39 | 4.9 | 80 | 2.5 | 119 | .8 | 168 | 1.3 |
| 40 | 2.1 | 81 | 21.0 | 120 | .5 | 169 | .5 |
| 41 | 30.1 | 82 | 12.2 | 121 | 7.9 | | |
| 42 | 1.6 | 83 | 3.7 | 122 | 3.1 | 191 | .6 |
| 43 | 3.4 | 84 | .8 | 123 | 14.8 | 192 | .9 |
| 44 | .4 | 85 | .5 | 124 | 3.1 | 194 | .7 |
| | | | | 125 | .7 | | |
| 51 | .7 | 91 | 4.0 | | | 205 | 1.0 |
| 52 | .4 | 92 | 2.7 | 133 | 1.7 | 206 | 2.9 |
| 53 | 5.5 | 93 | 8.0 | 134 | .8 | 207 | 1.1 |
| 54 | 2.7 | 94 | 1.2 | 135 | 2.1 | 208 | .5 |
| 55 | 4.9 | 95 | 6.2 | 136 | 11.3 | | |
| 56 | .6 | 96 | 2.8 | 137 | 5.5 | 219 | 2.9 |
| 57 | 2.2 | 97 | 1.2 | 138 | .7 | 220 | 3.2 |
| 59 | 1.7 | 100 | 1.3 | | | 221 | .6 |
| | | 103 | .4 | 147 | .5 | | |
| 65 | 2.5 | | | 148 | .4 | 231 | .4 |
| 66 | .6 | 104 | .4 | 149 | 1.6 | | |
| 67 | 9.1 | 105 | 2.0 | 150 | 1.3 | 245 | 1.2 |
| 68 | 5.8 | 106 | .7 | 151 | 66.6 | | |
| 69 | 100.0 | 107 | 3.6 | 152 | 9.2 | 259 | .4 |
| 70 | 5.5 | 108 | 2.3 | 153 | 1.0 | | |
| 71 | 1.0 | 109 | 15.0 | | | 273 | .7 |
| | | 110 | 1.8 | 163 | 1.6 | | |
| 77 | 4.0 | 111 | .6 | 164 | .6 | 288 | 1.2 |
| 78 | .7 | 117 | .4 | 165 | .4 | 289 | .4 |
| 79 | 4.3 | | | 167 | 1.2 | >PAUSE | |

MS #4




Mass spectrum #5
(23)


FRN 6006 SPECTRUM 211 RETENTION TIME 6.6
LARGST 4: 69.1,100.0 41.1, 80.3 296.1, 48.3 81.1, 43.4
LAST 4: 489.3, .3 490.3, 3.3 491.2, 1.3 492.2, .5
PAGE 2 Y = 1.00
100
80
60
40
20
0
340
360
380
400
420
440
460
480
100
80
60
40
20
0
500
520
540
560
580
600
620
640

MS #5

FRN 6006 SPECTRUM 211 RET. TIME = 6.6
>PAUSE

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 29 | 9.2 | 59 | 7.9 | 86 | 1.8 | 112 | 6.9 |
| 30 | .4 | 60 | .7 | 87 | 2.1 | 113 | 7.3 |
| 31 | 4.1 | 61 | .5 | 88 | .4 | 114 | 1.6 |
| 32 | 13.2 | | | 89 | .5 | 115 | 1.2 |
| | | 63 | .8 | | | 116 | 1.5 |
| 36 | .3 | 64 | .6 | 91 | 6.1 | 117 | 1.0 |
| 37 | .4 | 65 | 6.1 | 92 | 2.7 | | |
| 38 | .8 | 66 | 2.6 | 93 | 12.9 | 118 | .7 |
| 39 | 12.6 | 67 | 33.7 | 94 | 4.8 | 119 | 1.5 |
| 40 | 5.5 | 68 | 22.7 | 95 | 23.2 | 120 | 1.1 |
| 41 | 80.3 | 69 | 100.0 | 96 | 5.7 | 121 | 4.2 |
| 42 | 5.6 | 70 | 11.5 | 97 | 4.7 | 122 | 1.8 |
| 43 | 28.7 | 71 | 10.4 | 98 | 2.3 | 123 | 6.2 |
| 44 | 2.3 | 72 | 3.4 | 99 | 2.5 | 124 | 2.1 |
| 45 | 6.0 | 73 | 3.7 | 100 | 3.7 | 125 | 3.7 |
| 46 | .4 | 74 | .4 | 101 | 1.3 | 126 | 11.2 |
| 47 | .7 | 75 | .4 | 102 | .6 | 127 | 3.8 |
| | | | | 103 | .8 | 128 | 13.2 |
| 50 | .7 | 77 | 7.2 | | | 129 | 4.3 |
| 51 | 1.7 | 78 | 1.4 | 104 | .3 | 130 | 1.3 |
| 52 | 1.8 | 79 | 12.5 | 105 | 3.6 | 131 | .8 |
| 53 | 15.6 | 80 | 5.7 | 106 | .9 | | |
| 54 | 3.6 | 81 | 43.4 | 107 | 5.3 | 132 | 1.1 |
| 55 | 28.7 | 82 | 10.8 | 108 | 2.6 | 133 | 1.9 |
| 56 | 4.3 | 83 | 8.6 | 109 | 37.4 | 134 | 1.1 |
| 57 | 5.7 | 84 | 4.3 | 110 | 3.8 | 135 | 3.6 |
| 58 | 1.5 | 85 | 8.6 | 111 | 5.1 | 136 | 4.0 |
| | | | | | | <CONT> | |

MS #5

FRN 6006 SPECTRUM 211 RET. TIME = 6.6
>PAUSE

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 137 | 7.8 | 162 | 1.2 | 188 | 1.5 | 215 | .5 |
| 138 | 5.5 | 163 | 1.4 | 189 | .9 | | |
| 139 | 5.3 | 164 | 2.3 | 190 | 1.2 | 216 | .8 |
| 140 | 5.3 | 165 | 1.3 | 192 | 3.6 | 217 | .9 |
| 141 | 5.2 | 166 | 4.8 | 193 | 2.1 | 218 | 1.1 |
| 142 | 6.0 | 167 | 2.1 | 194 | 4.2 | 219 | 1.0 |
| 143 | 5.2 | 168 | 10.8 | 195 | 1.8 | 220 | 3.5 |
| 144 | 19.5 | 169 | 6.1 | 196 | 4.9 | 221 | 2.5 |
| 145 | 2.5 | 170 | 34.5 | 197 | 4.0 | 222 | 7.0 |
| | | 171 | 4.8 | 198 | 5.6 | 223 | 2.3 |
| 146 | 1.4 | 172 | 3.4 | 199 | 1.4 | 224 | 17.6 |
| 147 | 1.6 | 173 | 2.4 | 200 | 3.2 | 225 | 3.9 |
| 148 | 1.1 | | | 201 | 1.9 | 226 | 5.7 |
| 149 | 2.3 | 174 | .6 | | | 227 | 1.4 |
| 150 | 2.0 | 175 | .4 | 202 | 2.2 | 228 | 1.7 |
| 151 | 1.8 | 176 | 1.3 | 203 | .8 | 229 | .8 |
| 152 | 7.4 | 177 | 1.4 | 204 | 2.5 | | |
| 153 | 5.1 | 178 | 2.3 | 205 | 2.0 | 230 | .9 |
| 154 | 9.3 | 179 | 1.1 | 206 | 3.7 | 231 | .5 |
| 155 | 5.4 | 180 | 3.4 | 207 | 1.6 | 232 | 1.3 |
| 156 | 2.8 | 181 | 2.0 | 208 | 12.0 | 233 | 1.1 |
| 157 | 10.5 | 182 | 4.1 | 209 | 3.4 | 234 | 3.1 |
| 158 | 1.7 | 183 | 2.1 | 210 | 8.0 | 235 | 1.2 |
| 159 | 1.0 | 184 | 13.8 | 211 | 3.3 | 236. | 7.1 |
| | | 185 | 13.8 | 212 | 7.4 | 237 | 2.3 |
| 160 | 1.5 | 186 | 18.2 | 213 | 2.8 | 238 | 8.8 |
| 161 | .8 | 187 | 2.0 | 214 | 2.1 | 239 | 1.4 |
| | | | | | | <CONT> | |

MS #5

FRN 6006 SPECTRUM 211 RET. TIME = 6.6
>PAUSE

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 240 | 4.2 | 265 | 3.4 | 290 | 1.8 | 316 | 1.1 |
| 241 | 2.7 | 266 | 4.6 | 291 | 1.4 | 317 | .4 |
| 242 | 3.8 | 267 | 1.6 | 292 | 7.3 | 318 | 1.2 |
| 243 | .9 | 268 | 2.7 | 293 | 2.7 | 319 | 1.8 |
| | | 269 | 1.3 | 294 | 22.8 | 320 | 3.5 |
| 244 | 1.3 | 270 | 2.5 | 295 | 5.9 | 321 | 4.3 |
| 245 | .5 | 271 | .8 | 296 | 48.3 | 322 | 4.6 |
| 246 | 1.3 | | | 297 | 9.4 | 323 | 2.1 |
| 247 | .6 | 272 | .8 | 298 | 4.8 | 324 | 3.5 |
| 248 | 3.2 | 273 | .5 | 299 | 1.1 | 325 | 1.3 |
| 249 | 1.1 | 274 | 1.7 | | | 326 | 1.1 |
| 250 | 6.4 | 275 | .8 | 300 | .5 | 327 | .5 |
| 251 | 3.2 | 276 | 4.3 | 302 | .9 | | |
| 252 | 9.2 | 277 | 1.9 | 303 | .5 | 328 | 1.3 |
| 253 | 1.8 | 278 | 17.7 | 304 | 1.6 | 329 | .4 |
| 254 | 2.9 | 279 | 4.9 | 305 | 1.1 | 330 | 1.1 |
| 255 | .8 | 280 | 18.7 | 306 | 3.7 | 331 | .6 |
| 256 | 1.8 | 281 | 8.7 | 307 | 1.8 | 332 | 2.4 |
| 257 | .8 | 282 | 5.8 | 308 | 3.5 | 333 | .9 |
| | | 283 | 1.6 | 309 | 1.3 | 334 | 2.7 |
| 258 | .8 | 284 | 1.4 | 310 | 12.1 | 335 | 1.6 |
| 259 | .4 | 285 | .6 | 311 | 3.7 | 336 | 3.4 |
| 260 | 2.3 | | | 312 | 8.8 | 337 | 3.5 |
| 261 | 1.2 | 286 | .6 | 313 | 1.9 | 338 | 2.6 |
| 262 | 7.6 | 287 | .5 | | | 339 | 1.9 |
| 263 | 3.2 | 288 | 1.1 | 314 | 1.3 | 340 | 1.4 |
| 264 | 6.8 | 289 | 1.3 | 315 | .6 | 341 | .7 |
| | | | | | | <CONT> | |

MS #5

FRN 6006 SPECTRUM 211 RET. TIME = 6.6
>PAUSE

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 342 | .6 | 368 | .7 | 396 | .5 | 423 | .4 |
| 343 | .3 | 369 | .3 | | | 424 | .8 |
| 344 | 1.1 | | | 398 | .8 | 425 | .6 |
| 345 | .6 | 370 | .4 | 399 | .5 | | |
| 346 | 2.1 | 371 | .4 | 400 | 3.4 | 426 | .4 |
| 347 | .9 | 372 | 1.0 | 401 | 1.1 | 428 | .8 |
| 348 | 2.2 | 373 | .3 | 402 | 1.1 | 429 | .8 |
| 349 | .8 | 374 | .9 | 403 | 1.1 | 430 | 4.1 |
| 350 | 1.8 | 375 | .3 | 404 | 1.0 | 431 | 2.2 |
| 351 | 1.0 | 376 | 1.8 | 405 | .5 | 432 | 8.7 |
| 352 | 5.7 | 377 | .7 | 406 | 1.1 | 433 | 2.8 |
| 353 | 4.3 | 378 | 1.7 | 407 | .6 | 434 | 3.5 |
| 354 | 3.8 | 379 | .6 | 408 | .7 | 435 | 1.1 |
| 355 | 1.6 | 380 | 1.0 | 409 | .3 | 436 | .9 |
| | | 382 | .6 | 410 | .4 | 437 | .4 |
| 356 | .9 | | | | | | |
| 357 | .3 | 384 | .5 | 412 | .8 | 442 | .4 |
| 358 | .9 | 386 | .7 | 413 | .4 | 444 | .6 |
| 359 | .5 | 387 | .5 | 414 | 7.0 | 445 | .3 |
| 360 | 2.7 | 388 | 1.4 | 415 | 2.7 | 446 | 1.7 |
| 361 | 1.0 | 389 | .8 | 416 | 6.8 | 447 | 1.5 |
| 362 | 2.3 | 390 | 1.5 | 417 | 2.1 | 448 | 4.6 |
| 363 | 1.0 | 391 | .7 | 418 | 29.2 | 449 | 2.5 |
| 364 | 1.5 | 392 | 1.6 | 419 | 10.1 | 450 | 1.6 |
| 365 | .8 | 393 | .6 | 420 | 3.4 | 451 | .6 |
| 366 | .8 | 394 | .7 | 421 | 1.0 | 452 | .3 |
| 367 | .4 | 395 | .3 | 422 | .6 | | |

<CONT>

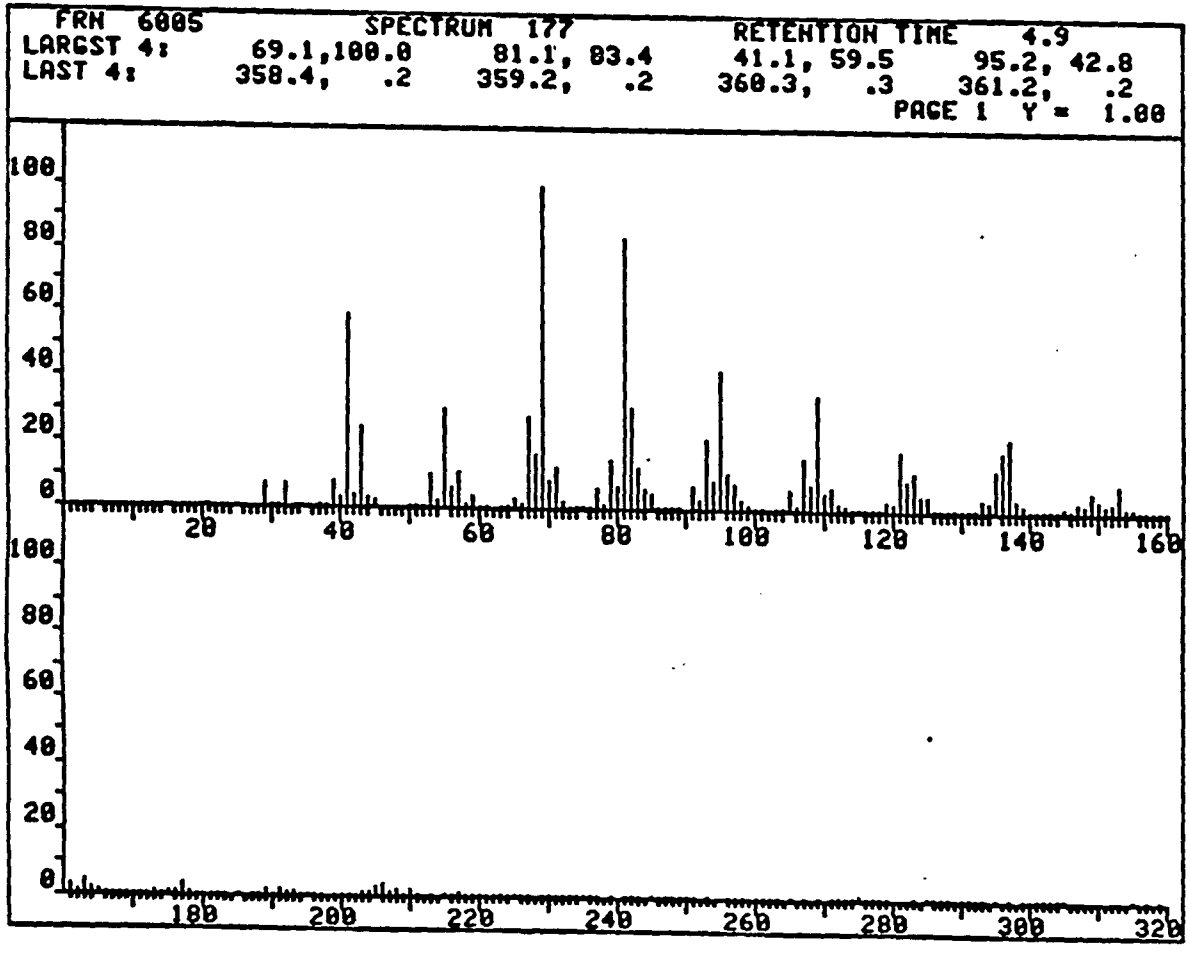
MS #5

FRN  6006 SPECTRUM  211 RET. TIME =  6.6

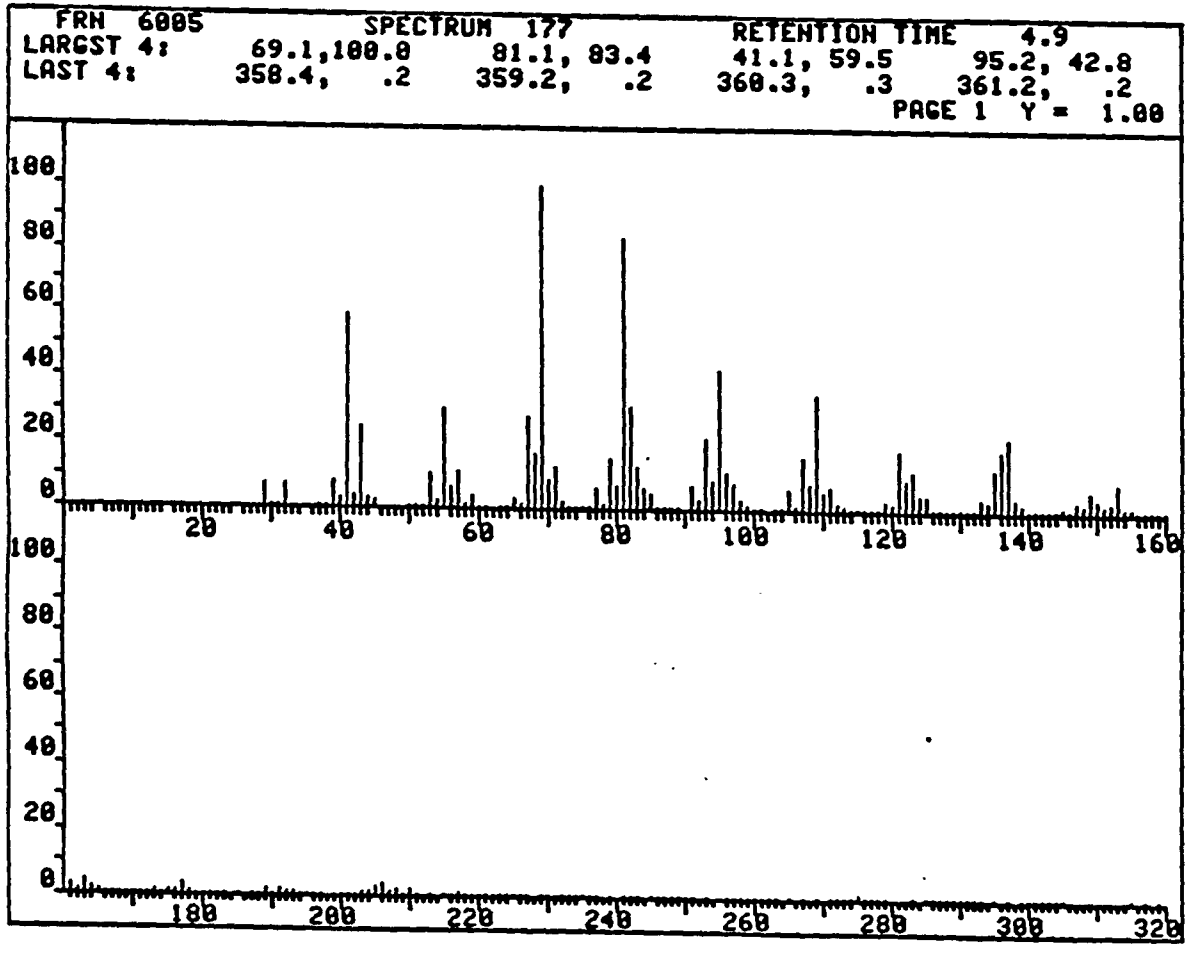
| MASS | ABUND |
|---|---|
| 459 | .3 |
| 460 | .4 |
| 461 | .3 |
| 462 | .5 |
| 463 | .5 |
| 464 | .5 |
| 465 | .3 |
| 472 | .8 |
| 473 | .3 |
| 474 | .3 |
| 476 | .3 |
| 486 | .5 |
| 488 | .9 |
| 489 | .3 |
| 490 | 3.3 |
| 491 | 1.3 |
| 492 | .5 |

>PAUSE

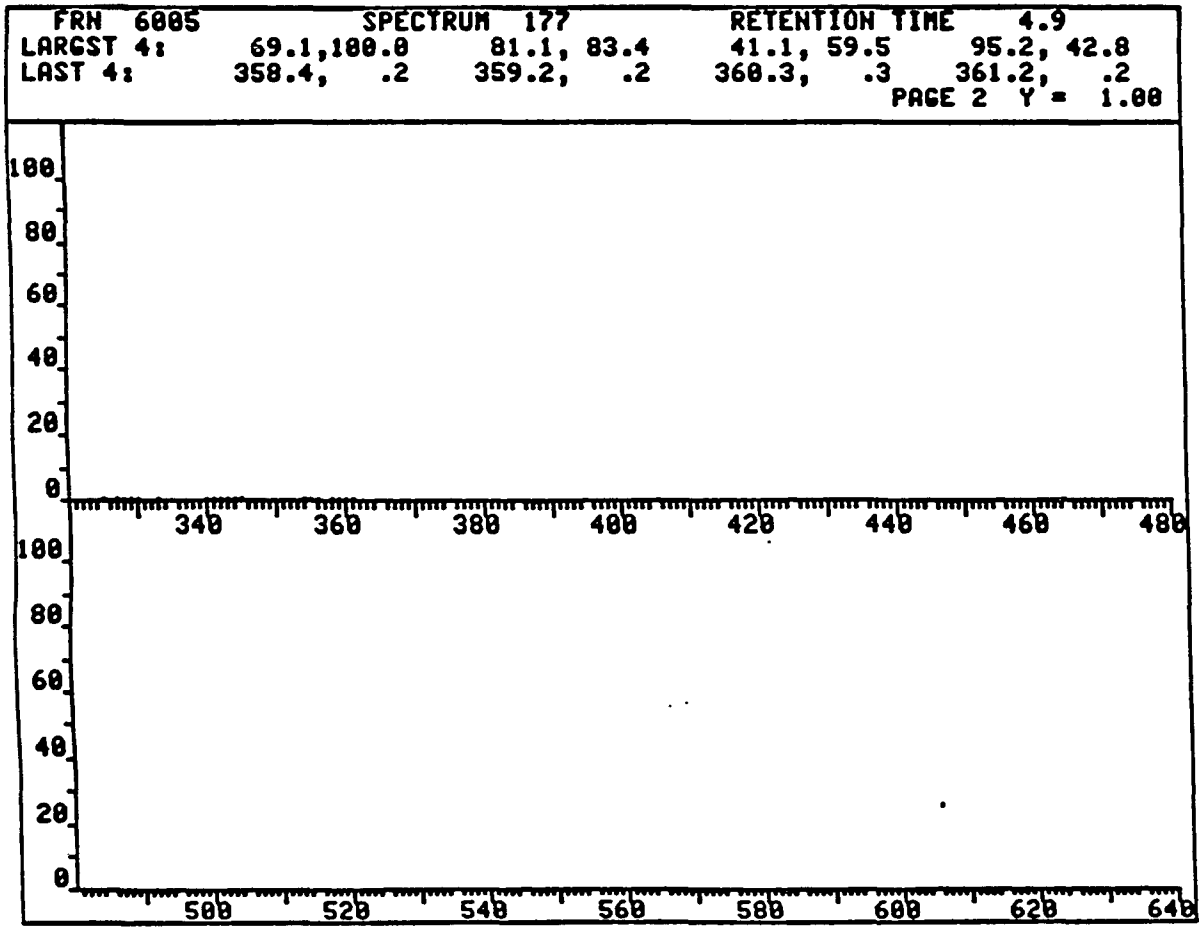
MS #5




Mass spectrum #6
(25)




Mass spectrum #6


FRN 6005 SPECTRUM 177 RETENTION TIME 4.9
LARGST 4: 69.1,100.0 81.1, 83.4 41.1, 59.5 95.2, 42.8
LAST 4: 358.4, .2 359.2, .2 360.3, .3 361.2, .2
PAGE 2 Y = 1.00
100
80
60
40
20
0
340
360
380
400
420
440
460
480
100
80
60
40
20
0
500
520
540
560
580
600
620
640

MS #6

FBN 6005 SPECTRUM 177 RET. TIME = 4.9
>PAUSE

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 29 | 7.0 | 63 | .2 | 89 | .2 | 116 | .3 |
| 30 | .3 | 64 | .2 | | | 117 | .7 |
| 31 | .9 | 65 | 3.8 | 91 | 7.4 | | |
| 32 | 7.1 | 66 | 1.5 | 92 | 3.2 | 118 | .3 |
| | | 67 | 28.2 | 93 | 22.0 | 119 | 3.4 |
| 37 | .1 | 68 | 17.6 | 94 | 8.7 | 120 | 2.1 |
| 38 | .3 | 69 | 100.0 | 95 | 42.8 | 121 | 18.8 |
| 39 | 7.8 | 70 | 8.9 | 96 | 11.2 | 122 | 9.7 |
| 40 | 3.2 | 71 | 13.2 | 97 | 8.2 | 123 | 12.3 |
| 41 | 59.5 | 72 | 2.2 | 98 | 2.9 | 124 | 5.1 |
| 42 | 4.1 | 73 | 1.0 | 99 | 2.0 | 125 | 4.7 |
| 43 | 25.1 | 74 | .1 | 100 | .4 | 126 | .9 |
| 44 | 2.8 | 75 | .2 | 101 | .3 | 127 | .7 |
| 45 | 2.2 | | | 103 | .4 | 128 | .7 |
| | | 76 | .1 | | | 129 | .9 |
| 50 | .2 | 77 | 6.7 | 104 | .3 | 130 | .1 |
| 51 | .8 | 78 | 1.4 | 105 | 6.3 | 131 | .9 |
| 52 | .8 | 79 | 15.5 | 106 | 2.0 | | |
| 53 | 10.3 | 80 | 7.5 | 107 | 15.8 | 132 | .8 |
| 54 | 2.4 | 81 | 83.4 | 108 | 8.1 | 133 | 4.2 |
| 55 | 30.6 | 82 | 31.4 | 109 | 35.3 | 134 | 2.9 |
| 56 | 6.3 | 83 | 12.8 | 110 | 5.6 | 135 | 12.9 |
| 57 | 11.2 | 84 | 6.1 | 111 | 7.3 | 136 | 18.4 |
| 58 | 1.4 | 85 | 4.9 | 112 | 2.3 | 137 | 23.0 |
| 59 | 4.2 | 86 | .5 | 113 | 1.4 | 138 | 4.0 |
| 60 | .3 | 87 | .4 | 114 | .2 | 139 | 2.6 |
| 61 | .2 | 88 | .1 | 115 | .5 | 140 | .9 |
| | | | | | | <CONT> | |

MS #6

FBH 6005 SPECTRUM 177 RET. TIME = 4.9
>PAUSE

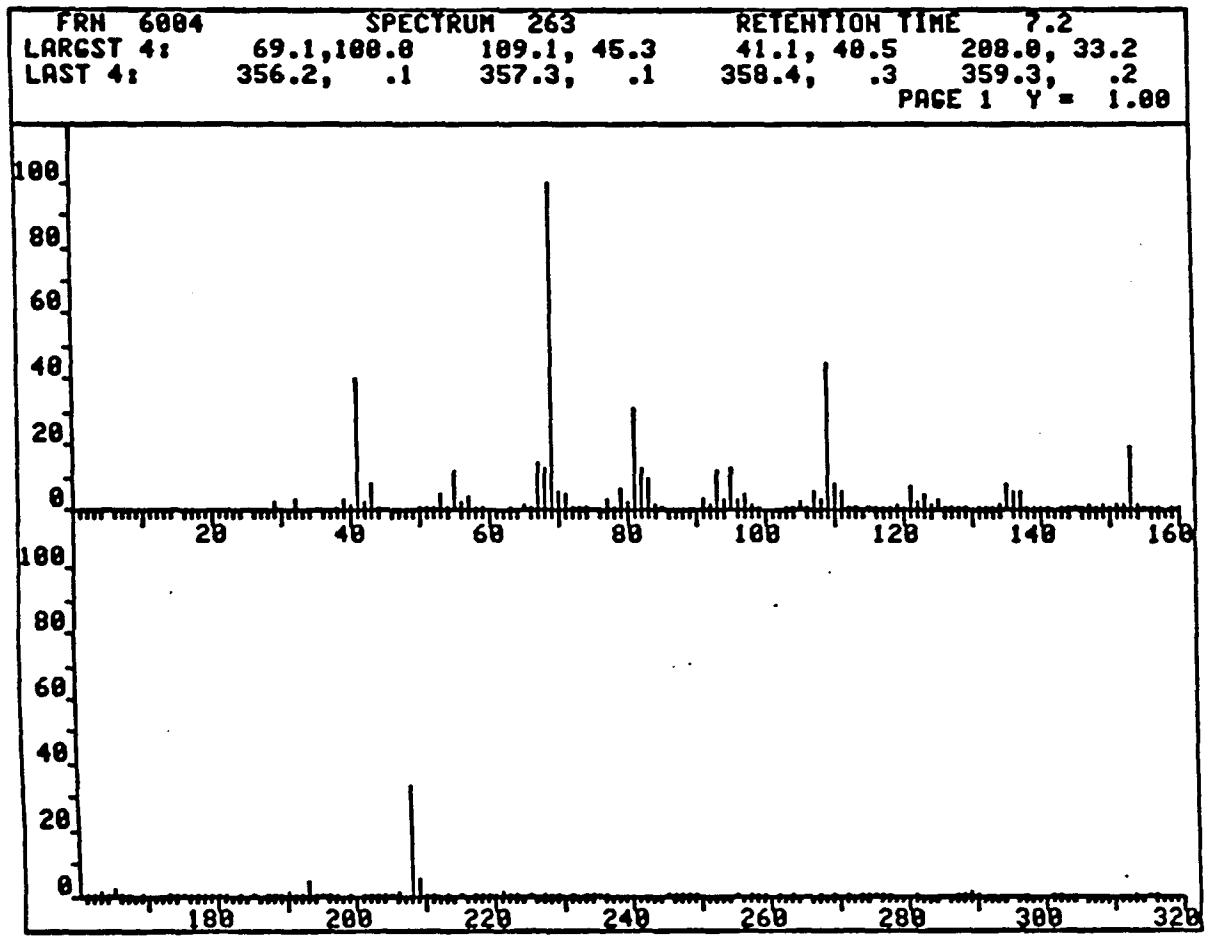
| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 141 | .7 | 166 | .6 | 193 | 1.4 | 221 | .8 |
| 142 | .3 | 167 | .6 | 194 | .4 | 222 | .4 |
| 143 | .7 | 168 | .4 | 195 | .5 | 223 | .4 |
| 144 | .3 | 169 | .8 | 196 | .3 | 224 | .2 |
| 145 | 1.4 | 170 | .3 | 197 | .2 | 225 | .2 |
| | | 171 | .4 | 199 | .2 | 226 | .1 |
| 146 | .5 | 172 | .1 | 200 | .1 | 227 | .1 |
| 147 | 2.8 | 173 | 1.0 | 201 | .6 | 229 | .4 |
| 148 | 2.6 | | | | | | |
| 149 | 6.7 | 174 | .2 | 202 | .4 | 230 | .2 |
| 150 | 4.3 | 175 | 1.5 | 203 | 1.4 | 231 | .6 |
| 151 | 2.6 | 176 | 1.0 | 204 | 1.2 | 232 | .4 |
| 152 | 3.4 | 177 | 4.2 | 205 | 2.5 | 233 | .5 |
| 153 | 8.9 | 178 | .9 | 206 | 4.2 | 234 | .3 |
| 154 | 1.9 | 179 | .5 | 207 | 1.4 | 235 | .4 |
| 155 | 1.4 | 180 | .5 | 208 | 1.8 | 236 | .3 |
| 156 | .3 | 181 | .4 | 209 | .6 | 237 | .3 |
| 157 | .3 | 182 | .1 | 210 | 2.3 | 239 | .1 |
| 158 | .2 | 183 | .4 | 211 | .4 | 241 | .2 |
| 159 | 1.0 | 185 | .4 | 213 | .3 | 242 | .1 |
| | | 187 | .5 | 215 | .5 | 243 | .2 |
| 160 | .4 | | | | | | |
| 161 | 2.9 | 188 | .4 | 216 | .4 | 245 | .4 |
| 162 | 1.4 | 189 | 2.1 | 217 | 1.3 | 246 | .2 |
| 163 | 4.8 | 190 | .8 | 218 | .7 | 247 | .2 |
| 164 | 1.8 | 191 | 1.9 | 219 | .5 | 248 | .2 |
| 165 | 1.4 | 192 | 1.4 | 220 | .7 | 249 | .4 |
| | | | | | | <CONT> | |

MS #6

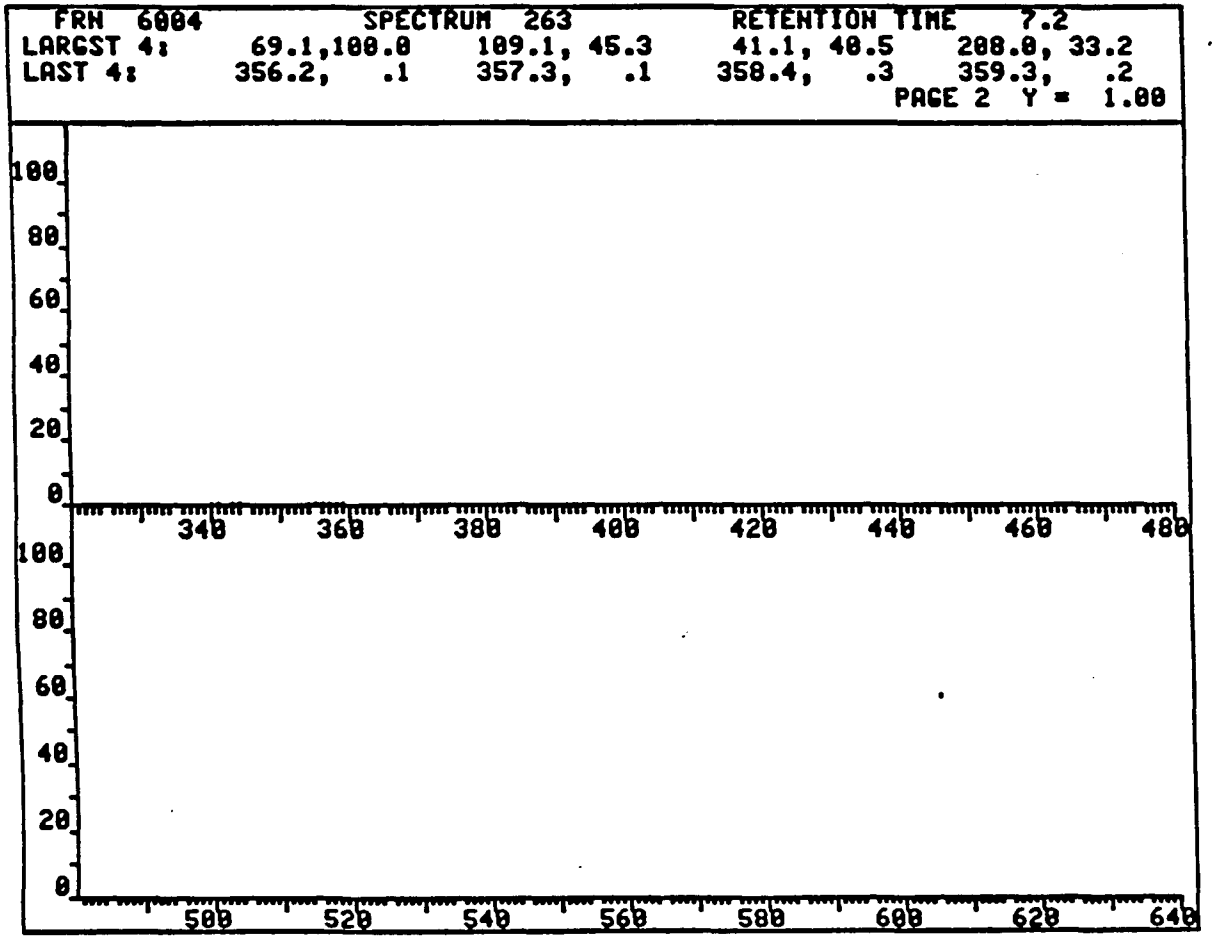
FRN 6005 SPECTRUM 177 RET. TIME = 4.9

| MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|
| 250 | .2 | 286 | .2 | 330 | .1 |
| 251 | .1 | 287 | .4 | 333 | .1 |
| 253 | .1 | 288 | .2 | 340 | .1 |
| 256 | .3 | 289 | .4 | 341 | .2 |
| 257 | .8 | 290 | .2 | | |
| | | 291 | .4 | 342 | .7 |
| 258 | .4 | 292 | .1 | 343 | .4 |
| 259 | .4 | 293 | .1 | 344 | .1 |
| 260 | .4 | 295 | .1 | 345 | .3 |
| 261 | .3 | 297 | .2 | 354 | .3 |
| 262 | .2 | 299 | .7 | 355 | .2 |
| 263 | .2 | | | | |
| 265 | .2 | 300 | .2 | 356 | .2 |
| 267 | .1 | 301 | .3 | 358 | .2 |
| 269 | .2 | 302 | .1 | 359 | .2 |
| 271 | .3 | 303 | .2 | 360 | .3 |
| | | 304 | .2 | 361 | .2 |
| 272 | .3 | 305 | .2 | >PAUSE | |
| 273 | .7 | | | | |
| 274 | .3 | 315 | .3 | | |
| 275 | 1.1 | 317 | .5 | | |
| 276 | .3 | 319 | .2 | | |
| 277 | .8 | 325 | .2 | | |
| 278 | .3 | 327 | .5 | | |
| 279 | .3 | | | | |
| 283 | .1 | 328 | .2 | | |
| 285 | .3 | 329 | .2 | | |

MS #6




Mass spectrum #7
(26)


FRN 6004 SPECTRUM 263 RETENTION TIME 7.2
LARGST 4: 69.1,100.0 109.1, 45.3 41.1, 40.5 208.0, 33.2
LAST 4: 356.2, .1 357.3, .1 358.4, .3 359.3, .2
PAGE 2 Y = 1.00
100
80
60
40
20
0
340
360
380
400
420
440
460
480
100
80
60
40
20
0
500
520
540
560
580
600
620
640

MS #7

>PAUSE

HN 6004 SPECTRUM 263 RET. TIME = 7.2

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 29 | 2.3 | 69 | 100.0 | 104 | .1 | 132 | .2 |
| 32 | 3.6 | 70 | 6.0 | 105 | 2.2 | 133 | 1.1 |
| | | 71 | 4.7 | 106 | .5 | 134 | 1.3 |
| 38 | .1 | 72 | .4 | 107 | 6.0 | 135 | 7.6 |
| 39 | 2.9 | 73 | .3 | 108 | 3.4 | 136 | 5.8 |
| 40 | 1.6 | 74 | .1 | 109 | 45.3 | 137 | 5.6 |
| 41 | 40.5 | | | 110 | 7.8 | 138 | 1.0 |
| 42 | 2.3 | 77 | 3.4 | 111 | 5.4 | 139 | 1.2 |
| 43 | 8.1 | 78 | .6 | 112 | 1.1 | 140 | .3 |
| 44 | .5 | 79 | 6.7 | 113 | .1 | 141 | .1 |
| | | 80 | 2.5 | 115 | .1 | 143 | .1 |
| 50 | .1 | 81 | 31.5 | 116 | .1 | 144 | .1 |
| 51 | .4 | 82 | 13.2 | 117 | .3 | 145 | .4 |
| 52 | .4 | 83 | 9.7 | | | | |
| 53 | 5.3 | 84 | 1.4 | 118 | .1 | 146 | .3 |
| 54 | 1.1 | 85 | .7 | 119 | 1.7 | 147 | 1.3 |
| 55 | 12.1 | | | 120 | 1.0 | 148 | 1.1 |
| 56 | 2.1 | 91 | 2.9 | 121 | 7.4 | 149 | 2.0 |
| 57 | 4.2 | 92 | 1.4 | 122 | 2.3 | 150 | 1.2 |
| 58 | .2 | 93 | 12.3 | 123 | 4.7 | 151 | 1.7 |
| 59 | .5 | 94 | 2.8 | 124 | 1.7 | 152 | 1.4 |
| | | 95 | 13.4 | 125 | 3.3 | 153 | 19.2 |
| 63 | .1 | 96 | 2.9 | 126 | .4 | 154 | 1.8 |
| 65 | 1.8 | 97 | 5.2 | 127 | .1 | 155 | .2 |
| 66 | .8 | 98 | 1.5 | 128 | .2 | 156 | .1 |
| 67 | 14.6 | 99 | .3 | 129 | .2 | 157 | .2 |
| 68 | 12.6 | 103 | .3 | 131 | .3 | 159 | .3 |

<CONT>

MS #7

FAN 6004 SPECTRUM 263 RET. TIME = 7.2
>PAUSE

| MASS | ABUND | MASS | ABUND | MASS | ABUND | MASS | ABUND |
|---|---|---|---|---|---|---|---|
| 160 | .2 | 192 | .3 | 223 | .1 | 273 | .5 |
| 161 | .9 | 193 | 5.8 | 225 | .1 | 274 | .2 |
| 162 | .4 | 194 | .8 | 227 | .1 | 275 | .8 |
| 163 | 1.1 | 195 | .2 | 228 | .1 | 276 | .3 |
| 164 | .4 | 196 | .1 | 229 | .2 | 277 | .1 |
| 165 | 1.8 | 197 | .1 | | | 281 | .1 |
| 166 | .5 | 199 | .1 | 231 | .2 | | |
| 167 | .2 | 201 | .3 | 233 | .4 | 287 | .2 |
| 169 | .1 | | | 234 | .1 | 288 | .1 |
| 173 | .3 | 202 | .2 | 235 | .1 | 289 | 1.1 |
| | | 203 | .5 | 236 | .1 | 290 | .3 |
| 174 | .1 | 204 | .7 | | | 297 | .1 |
| 175 | .9 | 205 | .7 | 245 | .2 | | |
| 176 | .4 | 206 | .9 | 246 | .1 | 301 | .1 |
| 177 | .7 | 208 | 33.2 | 247 | .2 | 302 | .1 |
| 178 | .2 | 209 | 5.1 | 248 | .1 | 313 | .1 |
| 179 | .3 | 210 | .7 | 249 | .2 | | |
| 180 | .4 | 211 | .1 | 255 | .1 | 315 | .6 |
| 181 | .1 | 213 | .1 | 257 | .2 | 316 | .1 |
| 183 | .1 | 215 | .2 | | | | |
| 185 | .1 | | | 258 | .1 | 340 | .2 |
| 187 | .2 | 217 | .2 | 259 | .1 | 341 | .1 |
| | | 218 | .1 | 261 | .1 | | |
| 188 | .2 | 219 | .2 | 263 | .1 | 343 | .2 |
| 189 | .6 | 220 | .2 | 267 | .1 | 344 | .1 |
| 190 | 1.1 | 221 | 1.3 | 269 | .1 | 354 | .1 |
| 191 | .4 | 222 | .4 | 271 | .1 | 355 | .2 |
| | | | | | | <CONT> | |

MS #7

FRM 6004 SPECTRUM 263 RET. TIME = 7.2

MASS · ABUND

| | |
|---|---|
| 356 | .1 |
| 357 | .1 |
| 358 | .3 |
| 359 | .2 |

>PAUSE

MS #7

## III. DISCUSSION

The mechanistic concept of regular terpene biosynthesis (i.e., head-to-tail polymerization) has not evoked much controversy in recent years, after the pioneering work of Cornforth,[34-40] Bloch and Popják. Conversely, the few exceptions to these head-to-tail terpenes have continuously plagued the bio-organic chemist. For example, artemisia ketone (I) which exhibits 4-2 dimerization of its two isopentane groups (see Fig. 3), not only possesses an irregular terpene skeleton, but biological studies[65-71] indicate that distinctly separate precursors may be involved. Analogously, bakuchiol (II), which exhibits the same quaternary carbon structure as artemisia ketone, cannot possibly arise from any pathway similar to regular terpene biosynthesis.

Indeed the most troublesome facet of irregular terpene biosynthesis is the formation of a carbon-carbon bond between two electrophilic carbons. As such, the polarity of one of these carbons must be reversed in order to couple the two terpenoid moieties. Since squalene, the most extensively studied irregular terpene, is formed similarly, the answer to other irregular terpenoid biosyntheses may be found in these investigations. Woodward[128,129] suggested that thiamine pyrophosphate is responsible for the necessary polarity reversal. Thus, upon alkylation of

thiamine with farnesyl pyrophosphate, abstraction of the C-1 proton, alkylation of another farnesyl moiety, and release of thiamine by reduction (Fig. 24) generates squalene.

This thiamine proposal was first experimentally tested by Bell[135] when he incubated citral, geraniol and thiamine in a cell-free yeast enzyme preparation. A new compound was isolated from the reaction mixture. Based on biogenic considerations, a structure (III in Fig. 25) was proposed. However, analysis of the mass spectrum invalidated this proposal. Karimian[126] then initiated a synthetic approach to the problem. Although he was unable to synthesize 2-hydroxygeranyl thiamine, he was able to make 2-(hydroxy-citronellyl) thiamine, 1, and showed that it could be incorporated into an artemisia ketone analogue (18a) in a cell-free enzyme preparation. This metabolite suggested that Bell's compound may have the corresponding diisopentenyl artemisyl structure (18). Thus, in extending these initial studies, three problems must be investigated: 1) that the compound isolated by Bell has an artemisyl skeleton; 2) that 2-hydroxygeranyl thiamine does actually participate in this conversion; 3) studies investigating the effect of 2-alkyl thiamine compounds such as 2-geranyl thiamine on the biosynthesis of artemisyl and squalene compounds should be initiated.

Of the many methods for characterizing Bell's diterpenoid, the total synthesis of 18 would unambiguously determine whether the proposed structure is correct. The artemisyl skeleton

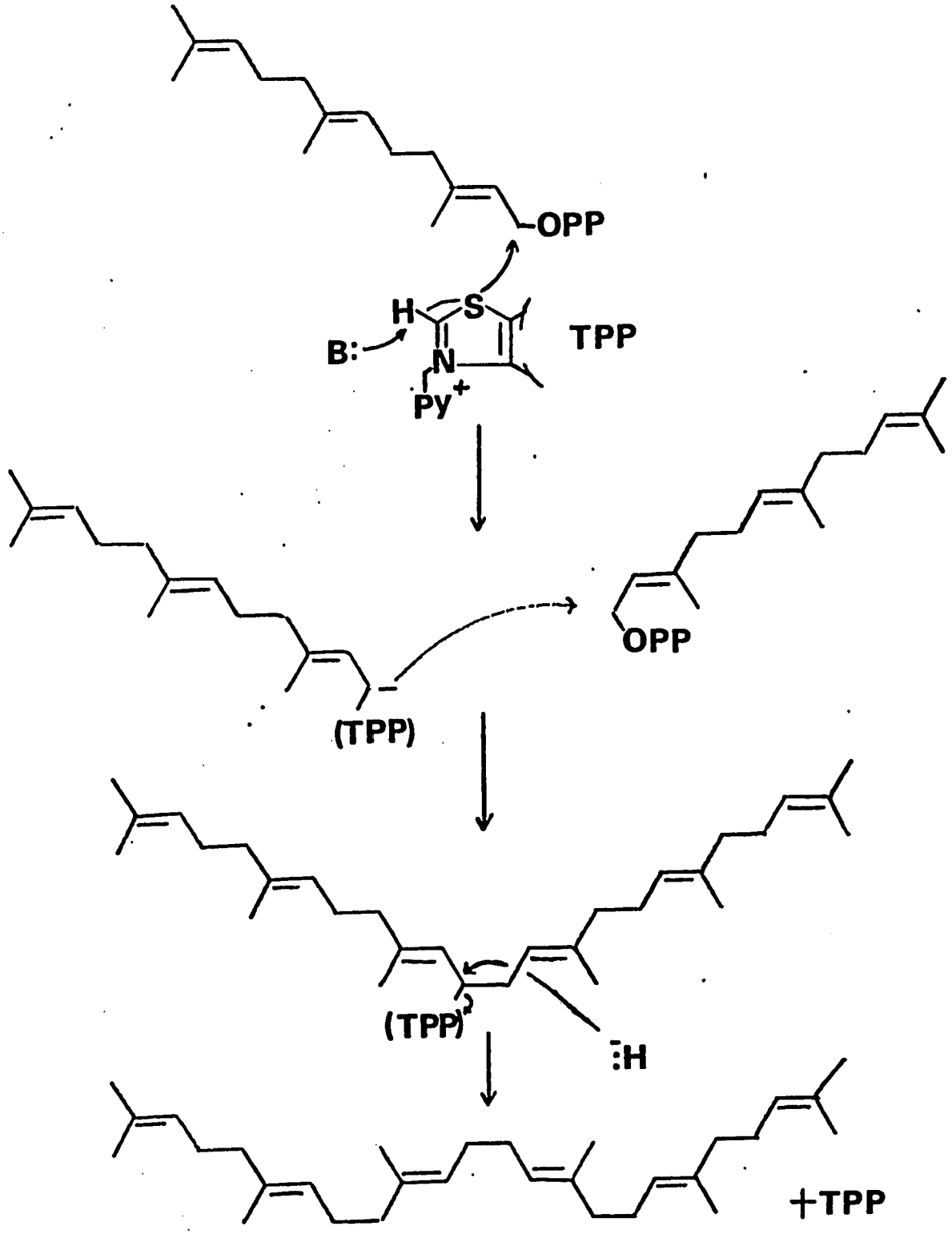
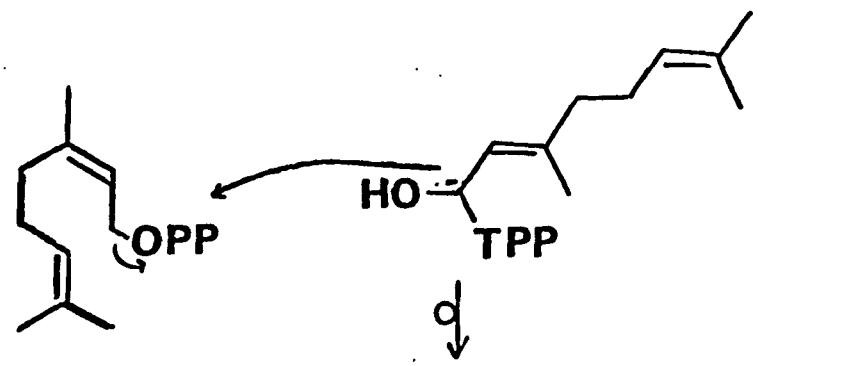
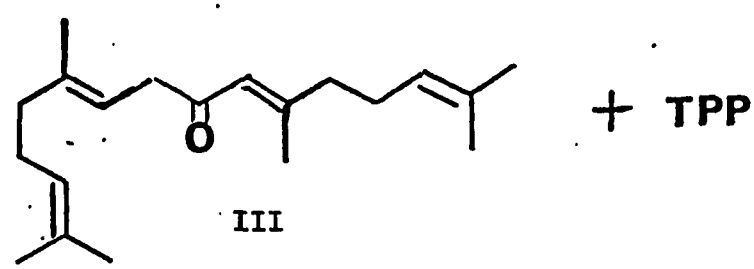
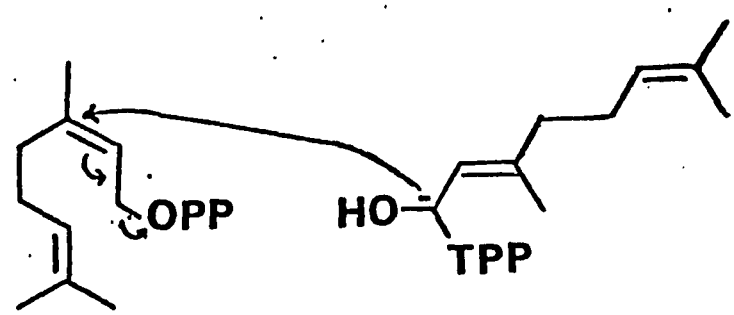


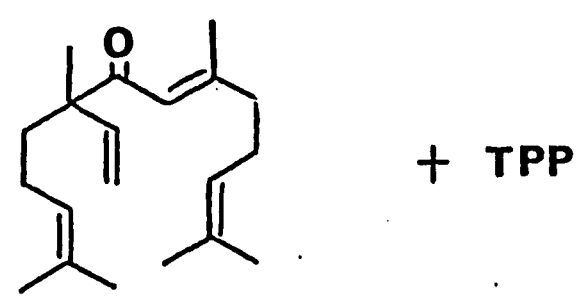

Figure 24. Woodward's proposed biosynthetic pathway.




III



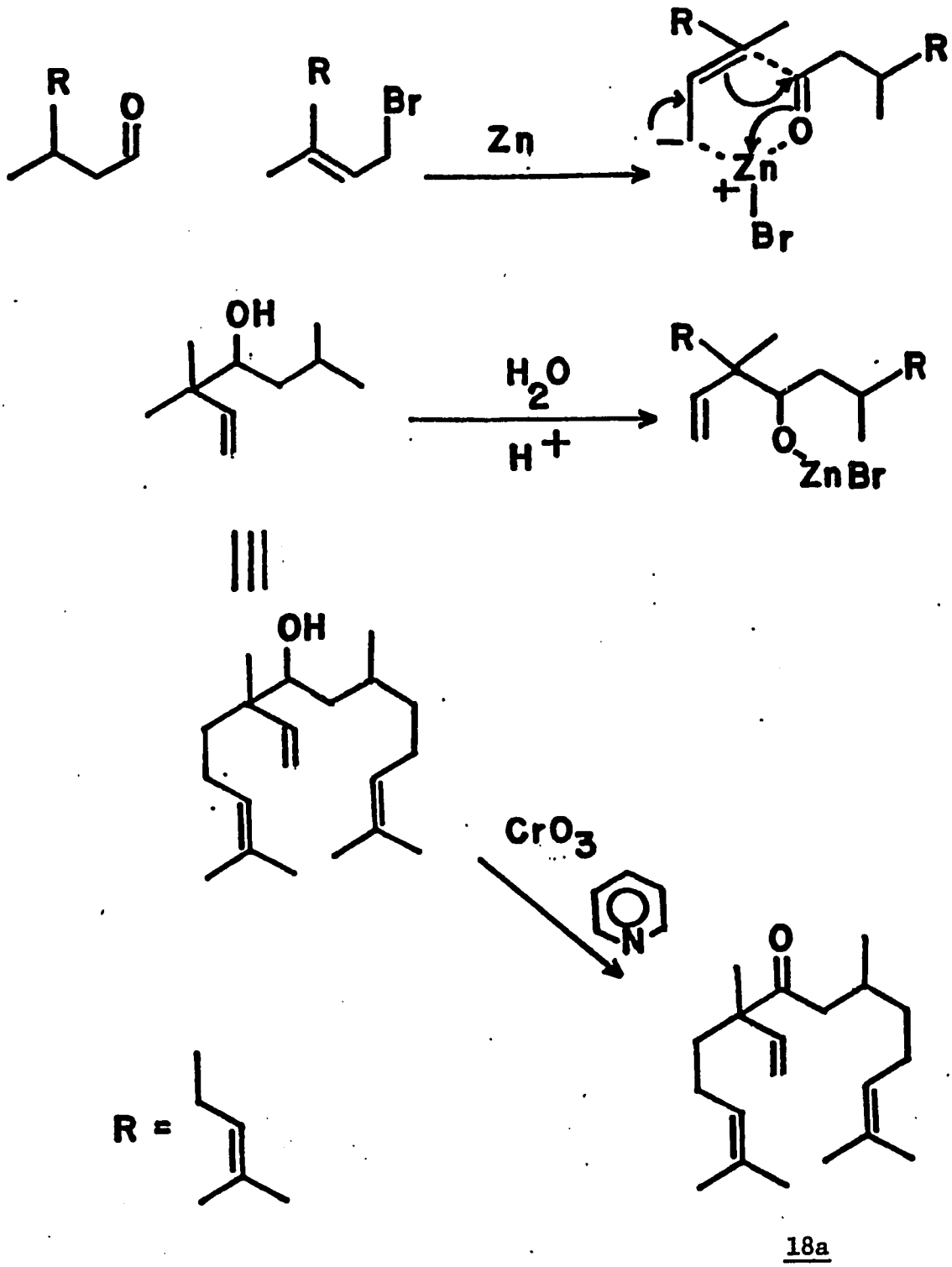




18

Figure 25. Bell's and Karimian's biosynthetic proposals for an irregular terpene isolated from yeast preparations.

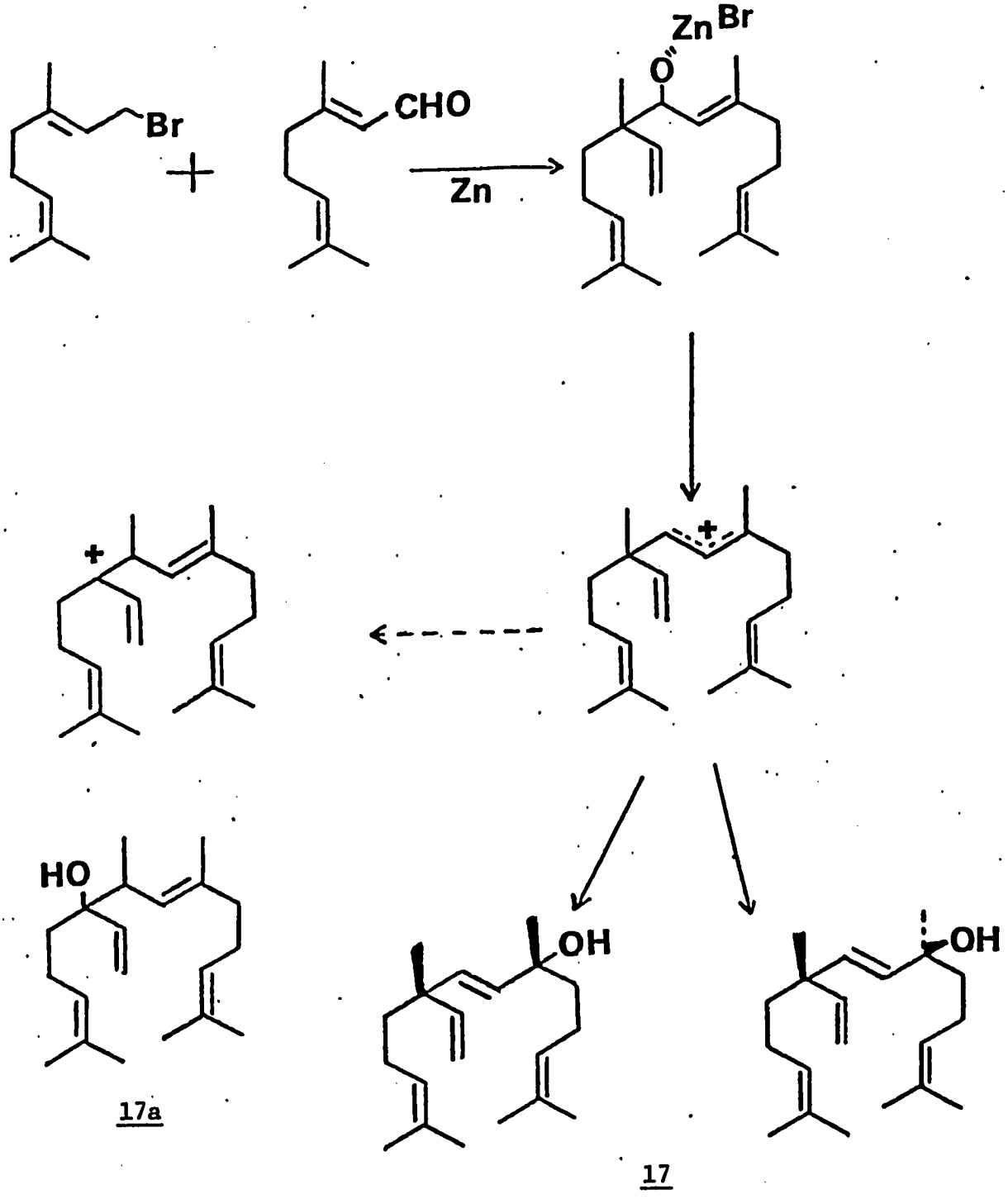
possesses a unique quaternary carbon structure in which there are two angular methyls and a terminal vinyl group attached to the α-carbon of the carbonyl. As a result of this relatively complex structure as well as the distinctive aroma of this irregular monoterpene, the total synthesis of artemisia ketone has attracted considerable attention from the organic chemist. Although a wide variety of methods have been developed for synthesizing artemisia ketone,[149,150,161-168] the superior synthetic scheme (Fig. 26) was the Continuous Flow Reformatsky Column.[149,150] This method has shown the most promise for several reasons. First, simple starting materials quickly and efficiently yield the desired products. Second, the quaternary center is formed in this reaction and requires no further modification. Third, the conditions are mild enough to ensure skeletal integrity. This synthetic method was successfully used by Karimian[126] to make 18a from geranyl bromide and citronellal. Carbon-carbon bond formation occurs through a six-membered ring intermediate (Fig. 26) wherein the carbanion generated attacks through the allylic position of the chelated carbonyl. This results in the alkylation by the third carbon (as opposed to the first) of the organometallic adduct. Subsequent oxidation with chromium trioxide gave the dihydro-artemisyl analogue, 18a.

This procedure seemed to be the simplest method for synthesizing diisopentenyl artemisia ketone. Thus, by substituting citral for citronellal and oxidizing with manganese dioxide, it was felt that this compound could be made. Geranyl bromide (Exp. 18)




18a

Figure 26. Mechanism of the Reformatsky reaction.

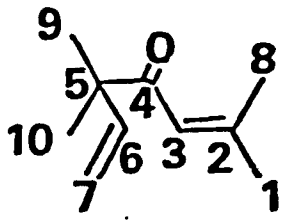



17a

17

Figure 27. Alternative proposals for the structure of 17.

and citral were reacted on the Reformatsky Column and a compound was obtained (17) which Karimian[126] found resisted oxidation. This was surprising, considering the relative ease of the formation of 18a. He explained this anomaly by invoking a 1,2-sigmatropic shift of the angular methyl at C-6. In this fashion, the doublet appearing at δ 0.9 in NMR 13 was accounted for. This methyl shift (Fig. 27) was thought to occur after solvolysis of the zinc-bromide alcohol. Subsequent hydrolysis of the resulting intermediate during the work-up generated 17a. Karimian's conclusions were based exclusively upon the lack of an oxidizable functionality and the unusual doublet appearing in the NMR. Upon review of similar reactions, this interpretation proved incorrect. As will be seen these two anomalies can be explained by an alternative proposal. Simpson[124] found that the same Reformatsky reaction, performed with dimethyl-allyl bromide and 3-methyl-2-butenal yielded 29% artemisia ketone (I) after oxidation. The remaining material proved to be yomogi alcohol (IV) (Fig. 28). In similar studies Thomas and Pawlak[169] attempted the syntheses of artemisia alcohol and ketone from yomogi alcohol and met with the same disappointing results. The opinion of these researchers was that the steric compression of the neopentyl alcohol in the artemisyl system makes this isomer unfavorable. To alleviate the strain between the alcohol and the substituents on the quaternary carbon, allylic rearrangement occurs. Thus, the more stable isomer is yomogi alcohol and artemisia alcohol cannot be isolated in appreciable yields.



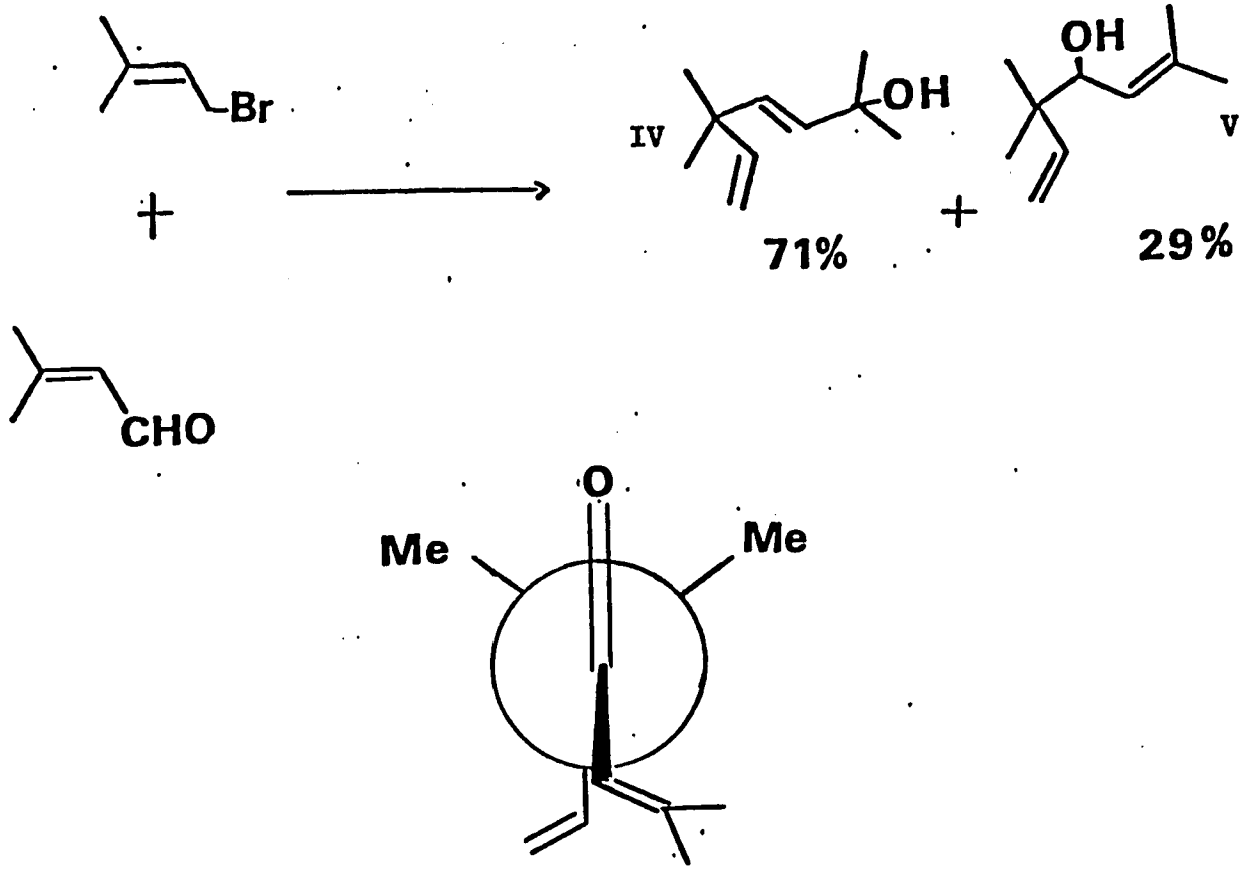




Figure 28. Isomerization of artemisia alcohol and Newman projection of artemisia ketone.

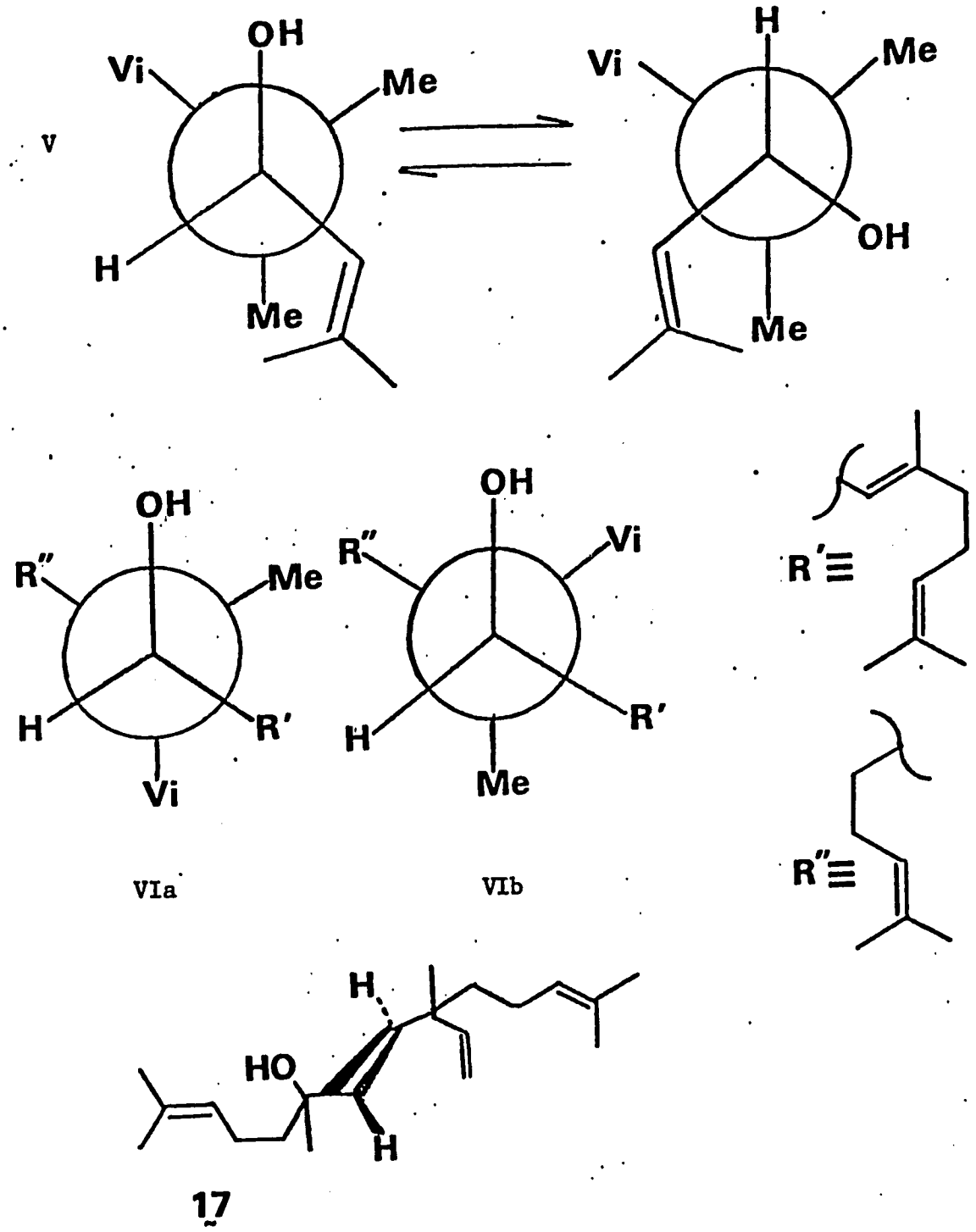



Figure 29. Comparison of artemisia alcohol and the twenty carbon analogue.

This explanation has merit. The upfield shift of the angular methyl of artemisia ketone (δ 1.0) can be attributed to shielding effects of the π cloud of the carbonyl oxygen[170] (Fig. 29). Clearly, only a closely aligned carbonyl group could influence the methyl in this fashion. If this steric effect was the sole factor influencing the ratio of products, then some diisopentenyl artemisia alcohol should be isolated. Since this was not the case, other factors must be involved.

The conformations of artemisia alcohol (V) and the diastereomers of the twenty carbon analogue (VIa and VIb) are compared (Fig. 29). The obvious difference is the increase of the steric bulk on C-1 and C-9 as a result of the extra isopentenyl groups. These larger groups not only restrict the rotation of the facial and eclipsed carbons shown, but add more steric strain to this system. If the facial carbon were $sp^2$ hydridized then this crowding would not occur because the large nine carbon group would be planar and would not influence the rest of the molecule sterically. Thus, allylic rearrangement of this intermediate occurs to alleviate the strain and the mixture of diasteromers produced would account for the methyl "doublets" appearing at δ 0.9 in NMR.

With the structure of 17 characterized, it was necessary to devise a strategy in which this compound could be converted to Bell's compound. Conversion of this yomogi alcohol to 18 would require migration of the olefin back to its original position and

oxidation at the secondary allylic carbon (Fig. 29). Such reactions are known and have been used successfully to convert linalool to citral.[171,172] In order to accomplish this oxidation, it was necessary to make the medium sufficiently acidic to generate the carbonium ion, yet mild enough to prohibit rearrangements and cyclizations through the other double bonds. For this reason, chromic acid in acetone, which has been described as a mild oxidizing agent,[151] was employed as the ideal reagent (Exp. 20). Upon isolation of the oxidized oil, no 18 was found. The most likely cause of this failure is, again, steric hindrance. Once the carbonium ion is formed, the three carbons forming this ion lie in one plane. In order to successfully oxidize this species, the chromate ion must attack the neopentyl position. Apparently, this ion is too large to accomplish this. Although chromate esters are formed from neopentyl alcohols (see Exp. 29), all have been formed by attack of the alcohol on chromic anhydride.[156] Thus, the energetics are great enough in the latter case to overcome the steric factors present in the artemisyl system (Fig. 30).

In the face of these difficulties, a slight change in strategy was needed. Since solvolysis of the zinc-bromide alcohol results in the formation of an analogue of yomogi alcohol, then avoiding this complication entails circumvention of the alcohol intermediate.

If the ketone could be generated directly as a result of the Reformatsky reaction, then the isomerization problem, encountered

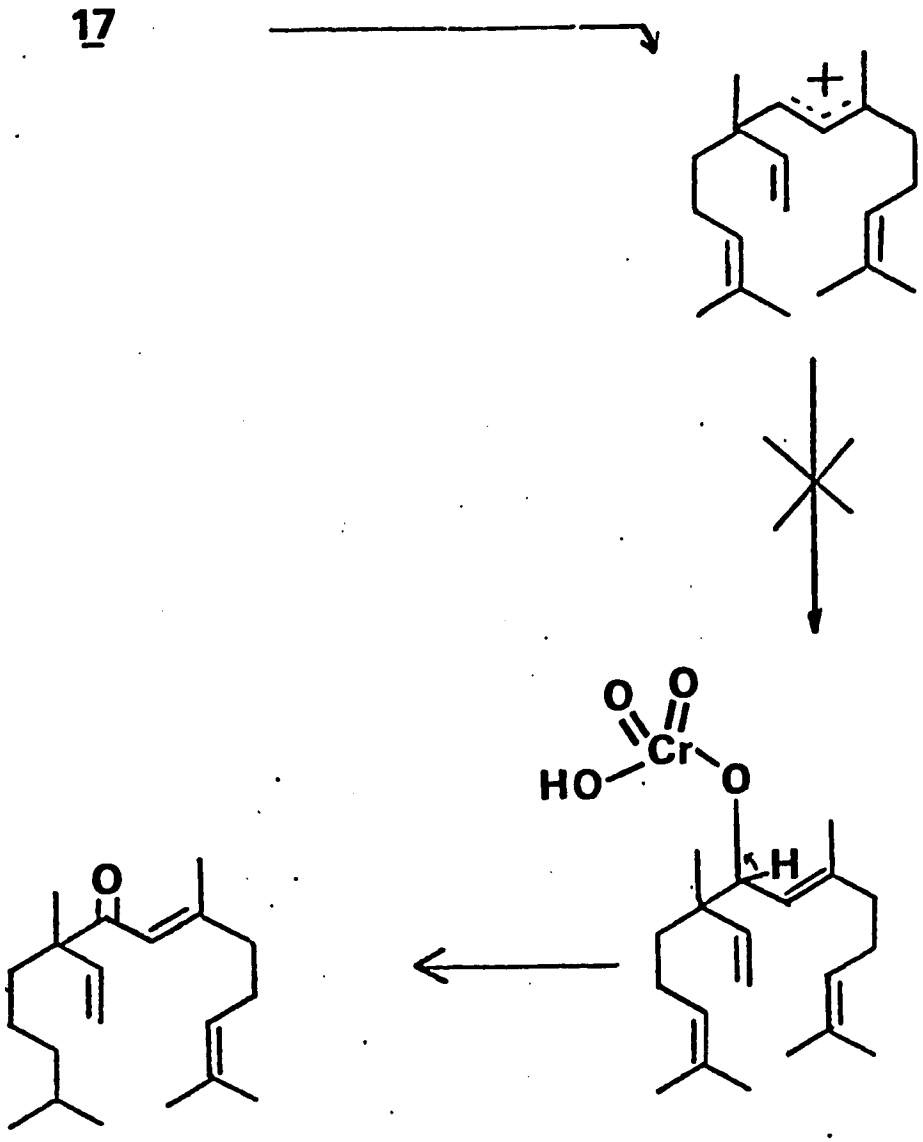



Figure 30. Attempted oxidation of 17.

previously, would be avoided. To produce a ketone directly the carbonyl bearing moiety must be an acyl derivative. Acid chlorides should be the most reactive compounds with organo-zinc derivatives, giving the highest yields (Fig. 31). However, considering the extreme reactivity of acyl halides, it was felt that some preliminary groundwork should be laid. In order to accomplish this, a model reaction was studied. Benzoyl chloride and geranyl bromide (Exp. 21) were reacted together on the zinc column. A phenyl analogue of artemisia ketone, 19, was isolated. Structure was confirmed by elemental analyses, NMR, IR, and mass spectral data. The upfield methyl spike at δ 1.1 (NMR 15) is characteristic of the angular methyl in the artemisyl skeleton. Aromatic signals show the classic "acetophenone" or multi-ordered AB split. The most conclusive evidence demonstrated by the NMR is the AA'B splitting of the terminal vinyl signal (δ 4.8-5.8). Corroborating this evidence is the strong absorption of an aromatic carbonyl at 5.9 microns (IR 8). The appearance of strong mass fragments at 137 m/e (geranyl), 105 m/e (benzoyl) and 69 m/e (isopentenyl) also demonstrate the validity of the proposed structure. It is important to note that the major cracks of this molecule (19) are analogous to those found by Bell for the irregular terpene isolated from the enzyme preparation.

The only drawback to this procedure is the low yields (54%) of the product. This is primarily due to the competing reaction between benzoyl chloride and the solvent, tetrahydrofuran (THF).

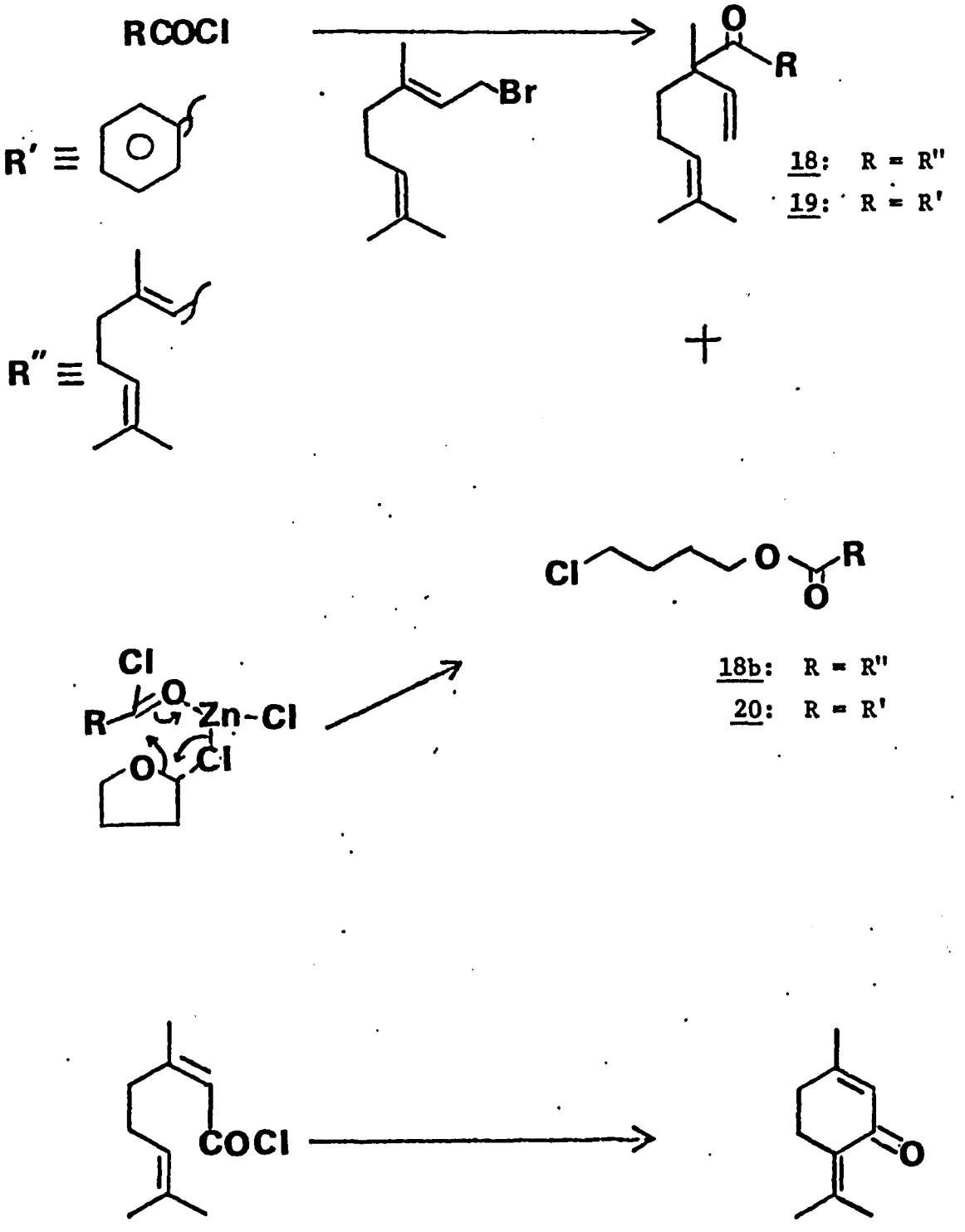



Figure 31. Reactions of acyl halides.

This reaction has been previously studied[157,173-175] (Exp. 22) and has been found to be catalyzed by metal halides, with zinc chloride being the best. The presence of zinc halide in the reaction is unavoidable; it is either a residue from the activation process or is formed by the Reformatsky reaction itself. The reaction presumably occurs through a six-membered transition state (Fig. 30). The zinc halide chelates with the carbonyl oxygen. Displacement of the ether oxygen by the chloride occurs after this oxygen attacks the carbonyl. The resulting adduct then releases the catalyst. THF was exceptionally reactive in this respect for two reasons. First, the cation generated on the δ-carbon of THF is stabilized in a non-classical fashion by the ether oxygen. Cyclic ethers would have less stability because two distinct species would be formed. Second, THF has the unique ability of solvating organo-zinc complexes.[176,177] If the zinc halides were less soluble, then this side reaction would be suppressed. Unfortunately, this latter factor is one of the principal reasons for the success of the Continuous Flow Reformatsky Column, for it was found that when diethyl ether or monoglyme was substituted, none of the desired Reformatsky product was obtained. It therefore seemed that this side reaction was inescapable.

Although recurrence of this reaction was annoying, the resulting chlorobutyl ester could easily be removed by preparative chromatography. Migration of this compound was consistently slower than the desired ketone. The next step was to synthesize 18.

The required acid was prepared by silver nitrate oxidation of citral in base (Exp. 23). Care must be taken to make sure that the glassware is meticulously clean, as impurities on the glassware reduce the yields rapidly. What proved to be the most difficult procedure was the synthesis of geranoyl chloride. In designing a strategem for its formation, conditions must be kept sufficiently mild to prevent decomposition of the acyl chloride by reaction with the isolated double bond. Piperitone (Fig. 31) has been synthesized by the reaction of geranoyl chloride in the presence of a Lewis acid.[178] Therefore, the reaction was performed in the presence of dimethyl formamide in an essentially basic medium (pyridine and benzene). DMF serves as a catalyst in this reaction; the dimethyl formamidoyl chloride, produced by the reaction of thionyl chloride and DMF, is the reactive species.[179-182] The imidoyl anhydride formed is broken by the subsequent addition of the chloride ion to the acyl carbonyl. DMF is thus regenerated.

With the acid chloride in hand, the synthesis of 18 was easily accomplished (Exp. 24). Again, low yields were obtained. The major contaminant in the product oil was the 4-chlorobutyl ester of geranic acid (see NMR 19). Final purification however resulted in the isolation of diisopentenyl artemisia ketone, 18, in 27% yield. Again, the upfield methyl spike (δ 1.1), the AA'B vinyl signal, the α-vinyl proton (δ 6.1) and the γ-carbon protons (downfield shifted to the carbonyl to δ 2.1-2.3) all confirm this structure.

The mass spectrum (MS 4) not only corroborates this conclusion, but shares many similar peaks (Table III) with the biologically prepared material, thus conclusively proving structure. Analysis of the fragments show that these molecular ions could only arise from the structure proposed for 18. McLafferty rearrangement[183,184] of the parent ion results in the formation of an ion at 206 m/e. If the same cleavage reaction occurs on the Cope rearrangement product (VII), then an ion at 220 m/e would result. Cleavage by α-fragmentation of (VIII) produces two ions: a geranyl radical-ion at 136 m/e and a citral signal at 151 m/e (IX). The large discrepancies in the amount of (IX) in the two mass spectra is probably due to further degradation of this ion in Bell's sample. Since the mass spectromoters were not the same,* such irregularities can be expected. However, further degradation of (IX) results in the formation of an ion at 123 m/e and carbon monoxide, thus confirming the presence of an ion at 151 m/e. Further degradation of the geranyl radical by demethylation gives an ion at 121 m/e. Cleavage of the rearranged product (X) results in a 93 m/e ion and ethylene. In both samples, isopentene (69 m/e) was the major cleavage fragment (Fig. 32).

If, as demonstrated, a compound with the peculiar structure characteristic of the artemisyl system was formed in the cell-free

---

*The biologically prepared material was analyzed on a Varian M66 Mass Spectromoter.

Table IV

| Biological (m/e) | Synthetic (m/e) |
|---|---|
| *220 | 288 |
| *206 | *220 |
| *136 | *206 |
| *123 | 151 |
| *121 | *136 |
| 111 | *123 |
| * 93 | *121 |
| 85 | 109 |
| 69 | * 93 |
| | 81 |
| | 69 |

Comparison of mass fragments of the biologically and chemically prepared sample of 18 (*indicates fragments common to both).

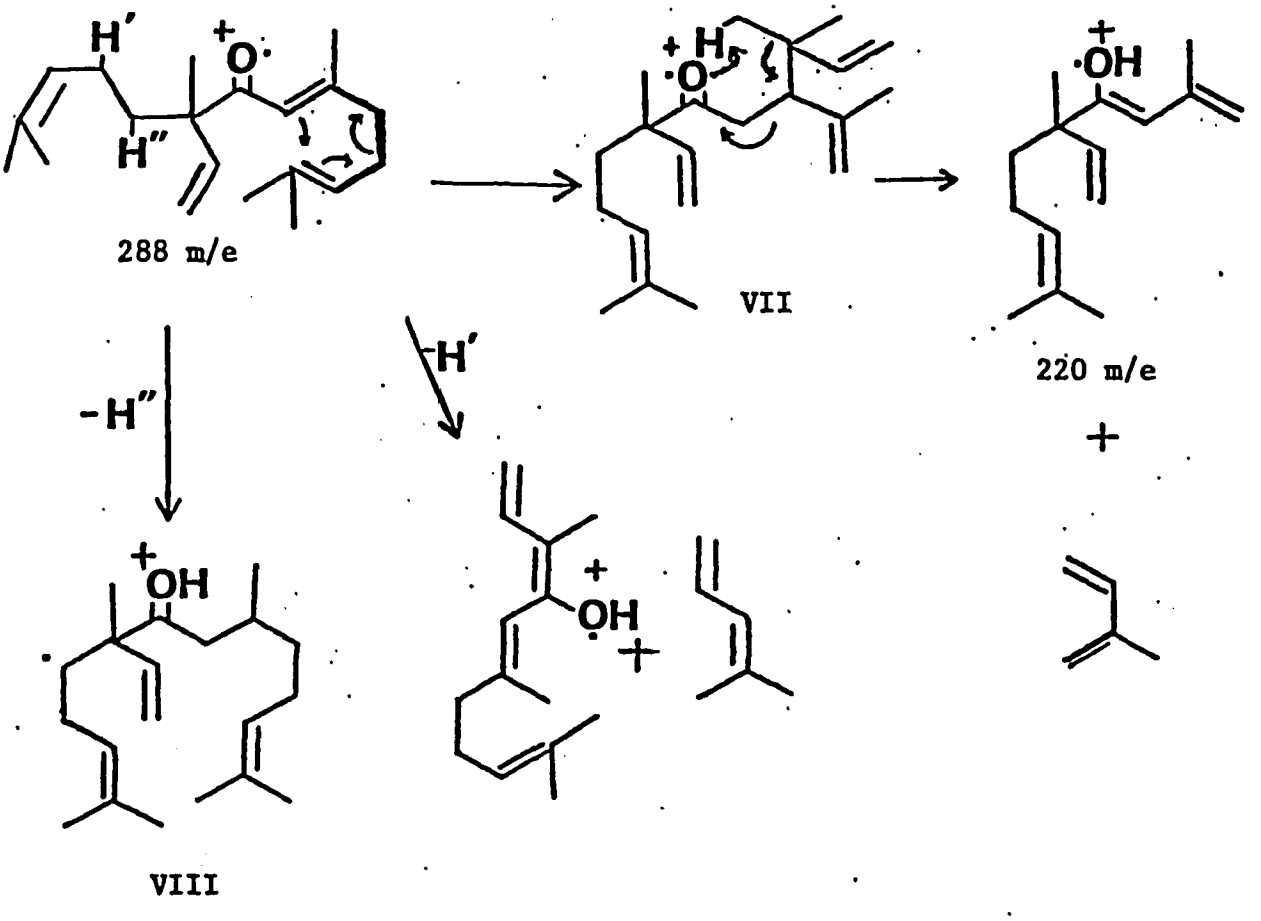



Figure 32. Mass spectral correlations of 18.

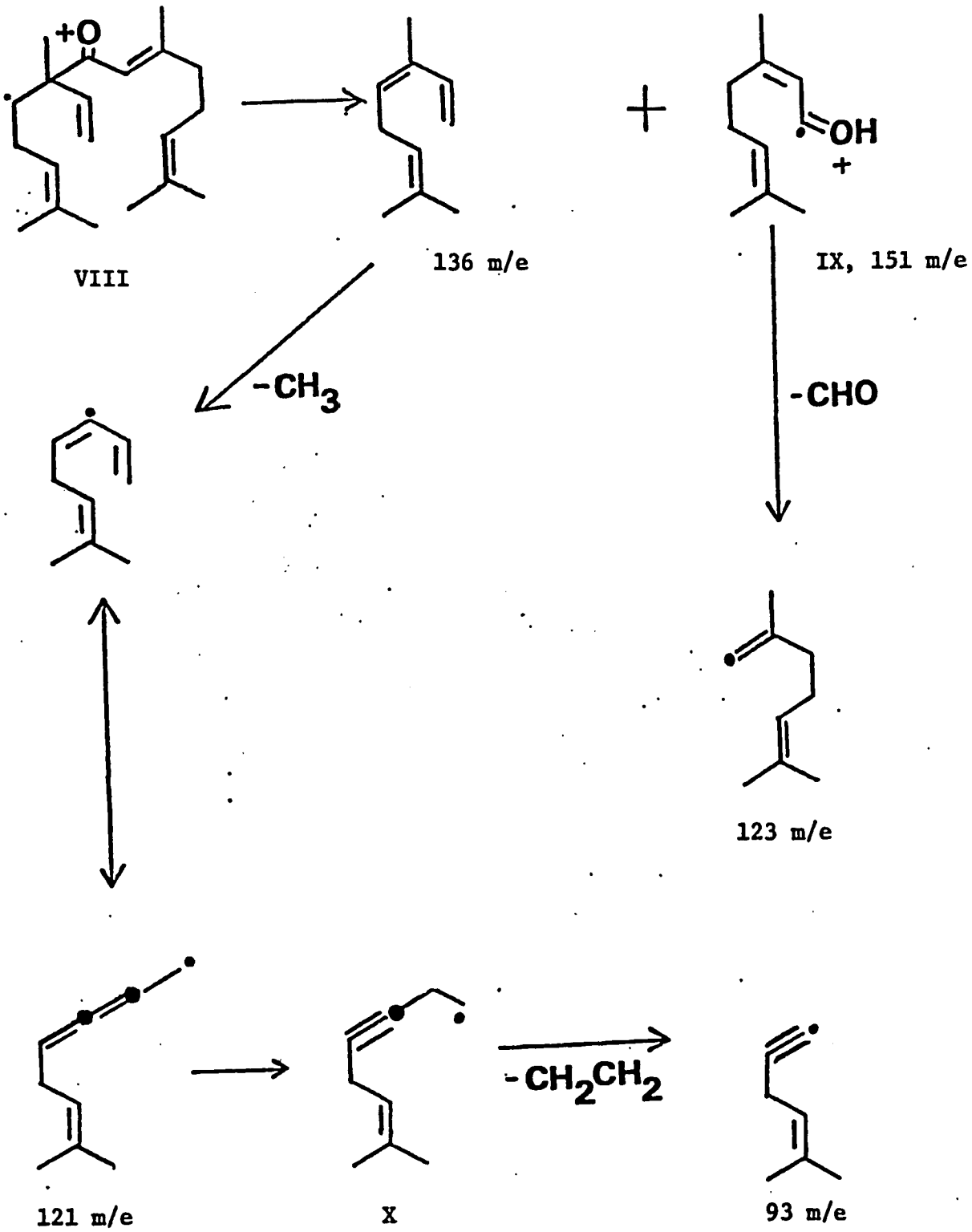



Figure 32 (continued).

yeast preparation, then a suitable mechanism regarding the biosynthesis of 18 needs to be determined. Although Bell[135] has shown that 18 was produced only after $B_1$ was added to the yeast preparation, it has yet to be demonstrated that thiamine is an obligatory cofactor for this conversion. To this end, the synthesis of 2-hydroxygeranyl thiamine was investigated. This compound should serve as the key intermediate in the formation of 18 (Fig. 33) by serving as an anionic nucleophile in an $S_N2'$ attack on geranyl pyrophosphate. Departure of the thiamine moiety would result in the formation of the diisopentenyl artemisia ketone, 18. Previous experimental results indicate that the reaction of α,β-unsaturated aldehydes and thiamine fails to yield the desired hydroxy-alkenyl derivative. This seems unusual in view of the facile reaction of saturated aldehydes and thiamine (e.g., Exp. 1 and 4).

This reaction usually occurs cleanly and with the formation of the desired hydroxyalkyl thiamine compound in high yield. The reaction is base catalyzed and is characterized by the presence of a lemon colored tinge. In contrast, the unsaturated aldehydes behave much differently. The reaction is dark brown and, upon acidification, no product is observed. Since the reaction between citral and thiamine would produce the desired compound directly, it was decided that the synthetic procedure should be studied more closely. In addition to the unusual behavior of these unsaturated aldehydes with thiamine, the formation of perhydrofuro derivatives from saturated analogs of thiamine in aprotic solvents, and the

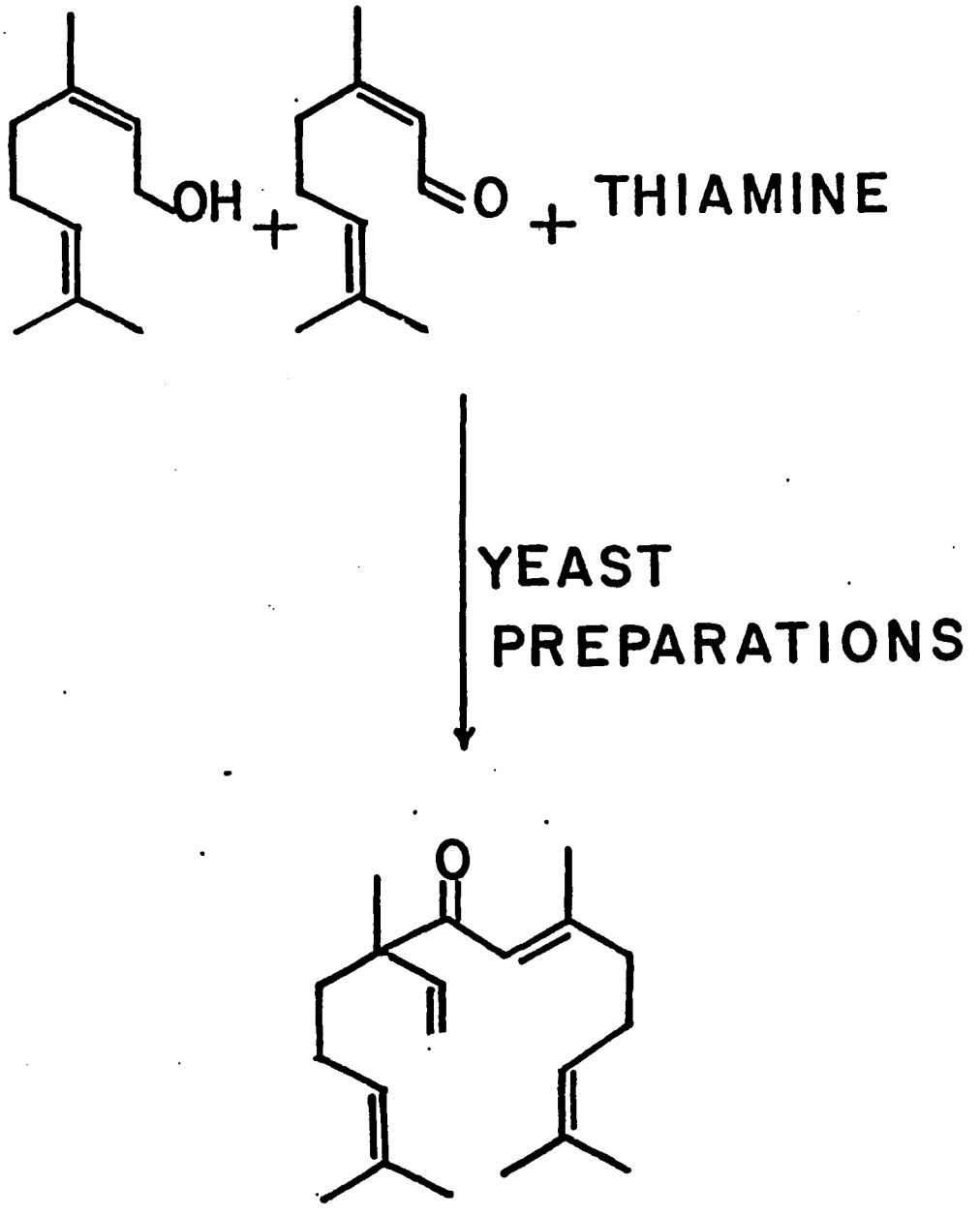



Figure 33. Biosynthesis of 18; Bell's experiment.

release of the hydroxy-alkyl side chains in protic solvents must be studied. The behavior of thiamine and aldehydes in this apparently capricious nature is similar to the behavior of carbenes under various conditions. It has been shown that (XI) will react with alcohols as shown in Fig. 34, but in the presence of aldehydes, these carbenes will yield the corresponding ketones.[185] As such, model studies using simple benzothiazolium salts (Fig. 34) have shown that aldehydes combine with these salts to form benzothiazolinyl ketones.[143] Thus, 3-methylbenzothiazolium hemisulfate, 7 (Exp. 8), and benzaldehyde were reacted in triethylamine and methanol to form 8, 3-methylbenzothiazolinyl phenyl ketone. The mechanism of this transformation has also been demonstrated to proceed through a carbene intermediate.[143,186,187] It has been proposed that the C-2 hydrogen of the thiazolium salts is abstracted directly to generate either the carbene[186,187] or the ylid.[188,189] There are two pitfalls for this mechanism. First, the apparent nucleophilicity of benzothiazolium salts is not consistent with simple base abstraction of the proton. It has been demonstrated[190,191] that 7 (Fig. 34) can be attacked by hydroxide to form the amide-mercaptide. This reaction is inconsistent with the electron rich carbon required at C-2 to make the proton acidic. Second, 2-hydroxydithienes[185] are excellent carbene precursors, yet do not possess $sp^2$ hybridization. It appears, therefore, that thiazolium salts are attacked by base to form 2-hydroxythiazolines which decompose to the carbene. Deprotonation of the 2-hydroxythiazoline can be accepted after considering

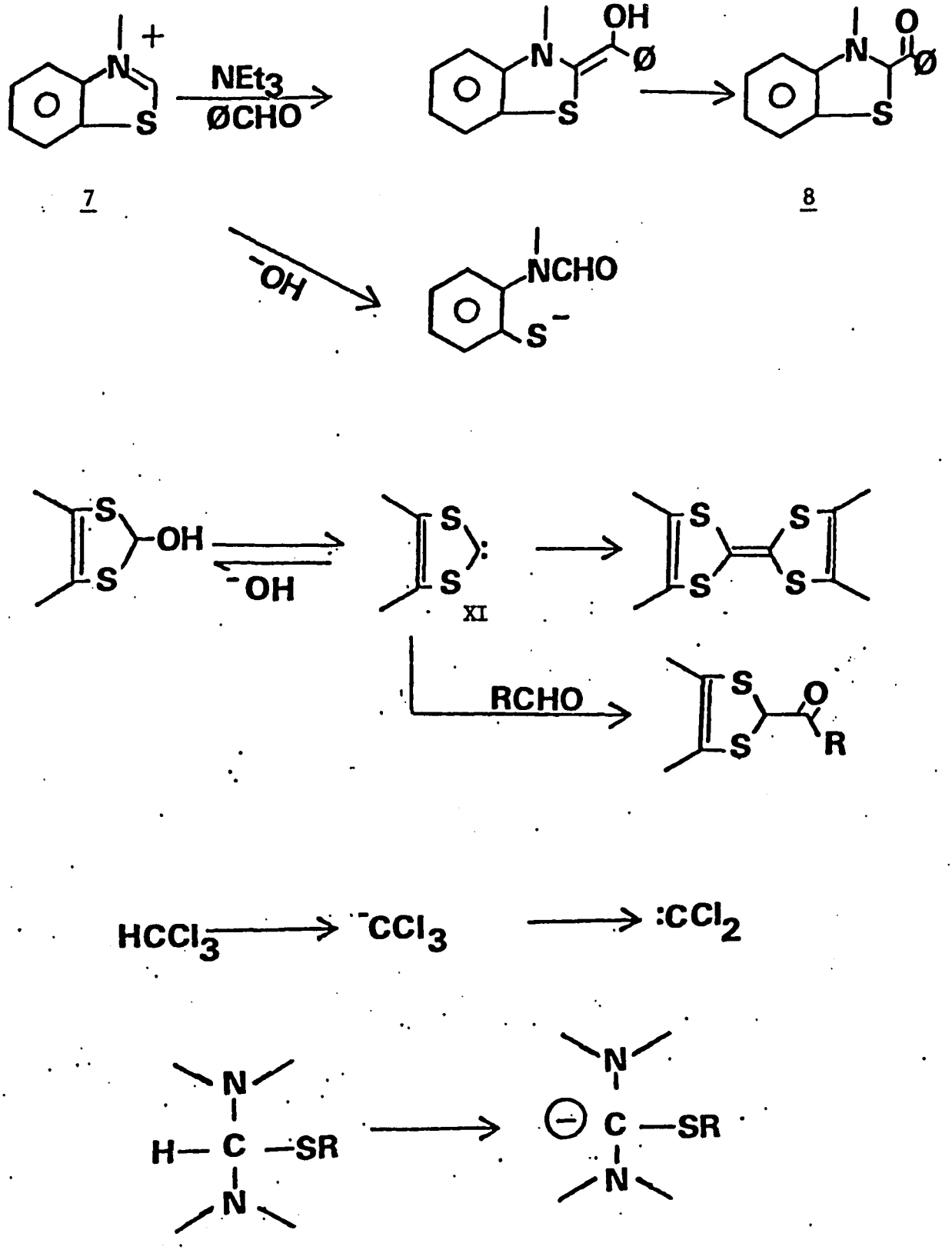



Figure 34. Carbenoid model systems.

other similar chemical systems. Chloroform[192-194] is deprotonated in base to form a stable intermediate, the trichlorocarbanion. Its stability is so great that the rate limiting step in the formation of dichlorocarbene is E-1 departure of the chloride ion.[193-198] Other systems containing nitrogen and sulfur heteroatoms[199] easily deprotonate under basic conditions.

Considering the wealth of information concerning carbenoid heterocycles, it is astonishing to find continued acceptance of the Breslow ylid of thiamine. It is immediately apparent that the chemistry of thiamine is governed by this principle. As such, the reaction of benzaldehyde with thiamine should be identical as that with benzothiazolium salts. Pursuant to this aim, the vitamin was reacted with benzaldehyde under base conditions (Exp. 6). The very fact that 5, the phenyl thiamine ketone, was isolated demonstrates the validity of the carbene mechanism as applied to thiamine.

Thus, the apparent arbitrary action of $B_1$ of aldehydes (Fig. 35) can be explained as follows: generation of the carbene must occur first by deprotonation of an intermediate having three hetero atoms attached to C-2 of the thiazole (Fig. 35). The yet unexplained purpose of the amino group of the pyrimidine is now clarified. Metzler[200,201] has shown that thiamine exists as the tricyclic form at physiological pH. The relative ease of the formation of thiochrome[202] (Fig. 36) substantiates the existence of this intermediate. Thus, as with other carbenoid precursors,

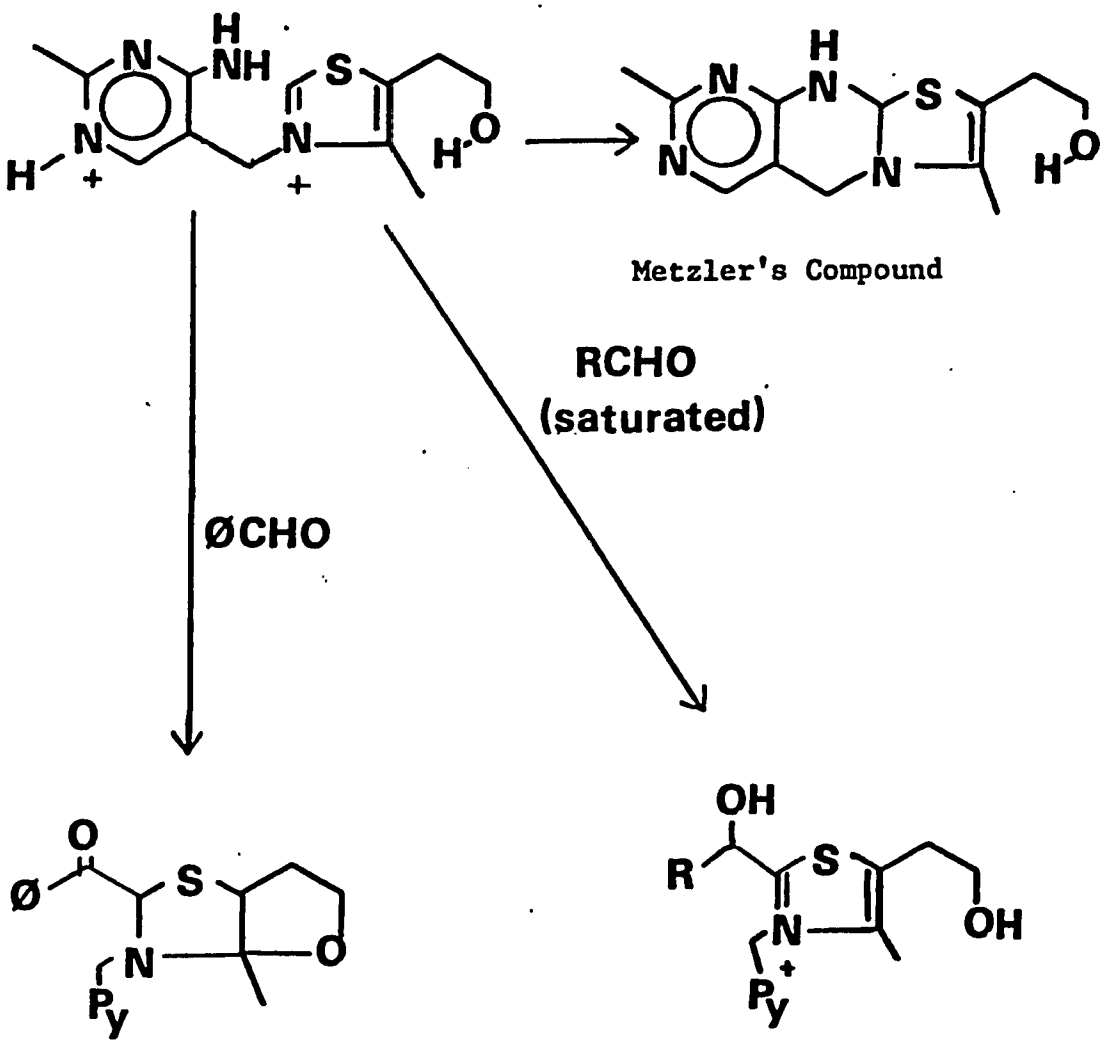



Figure 35. Reactions of thiamine.

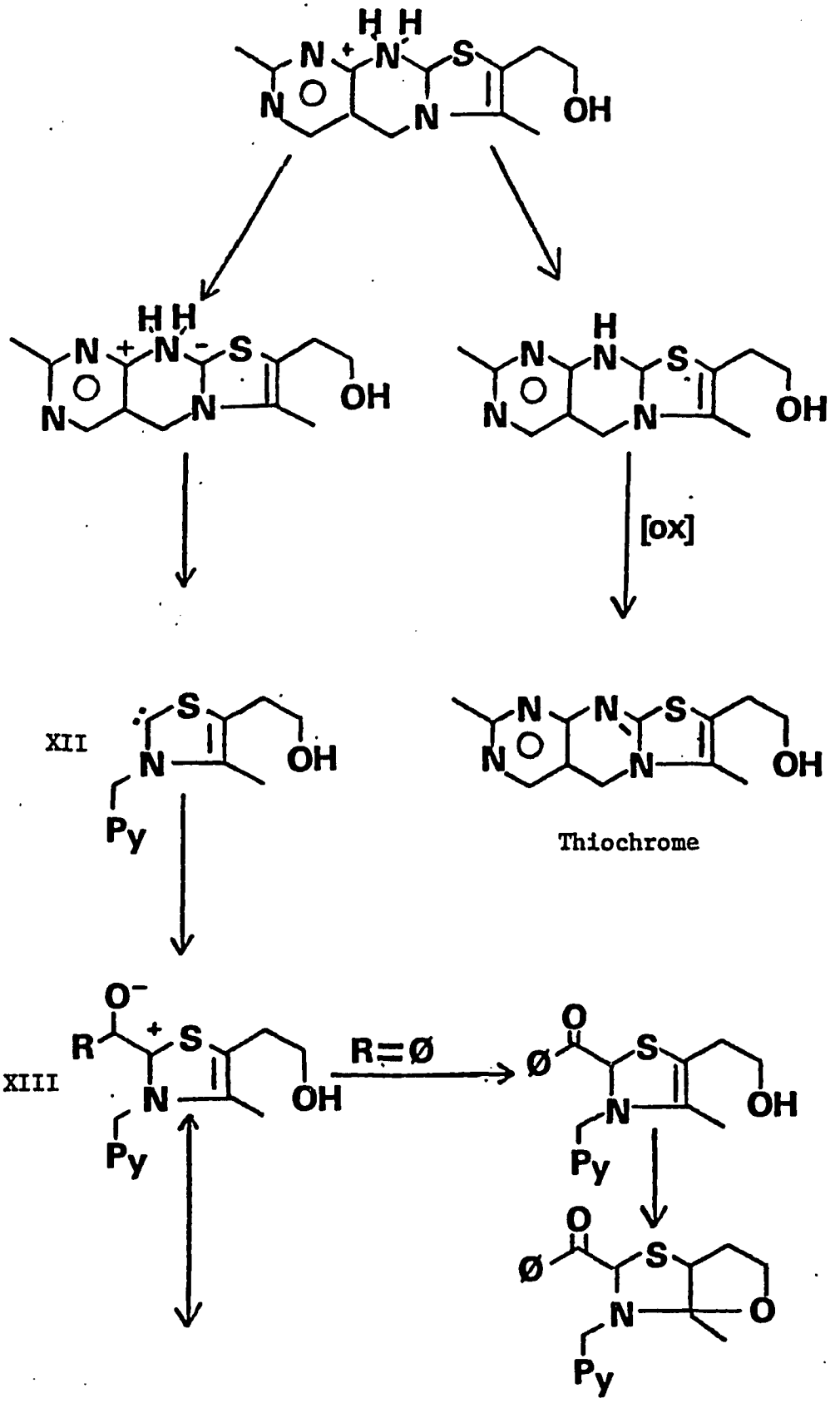



Figure 36. Proposed mechanism of thiamine via a carbene intermediate.

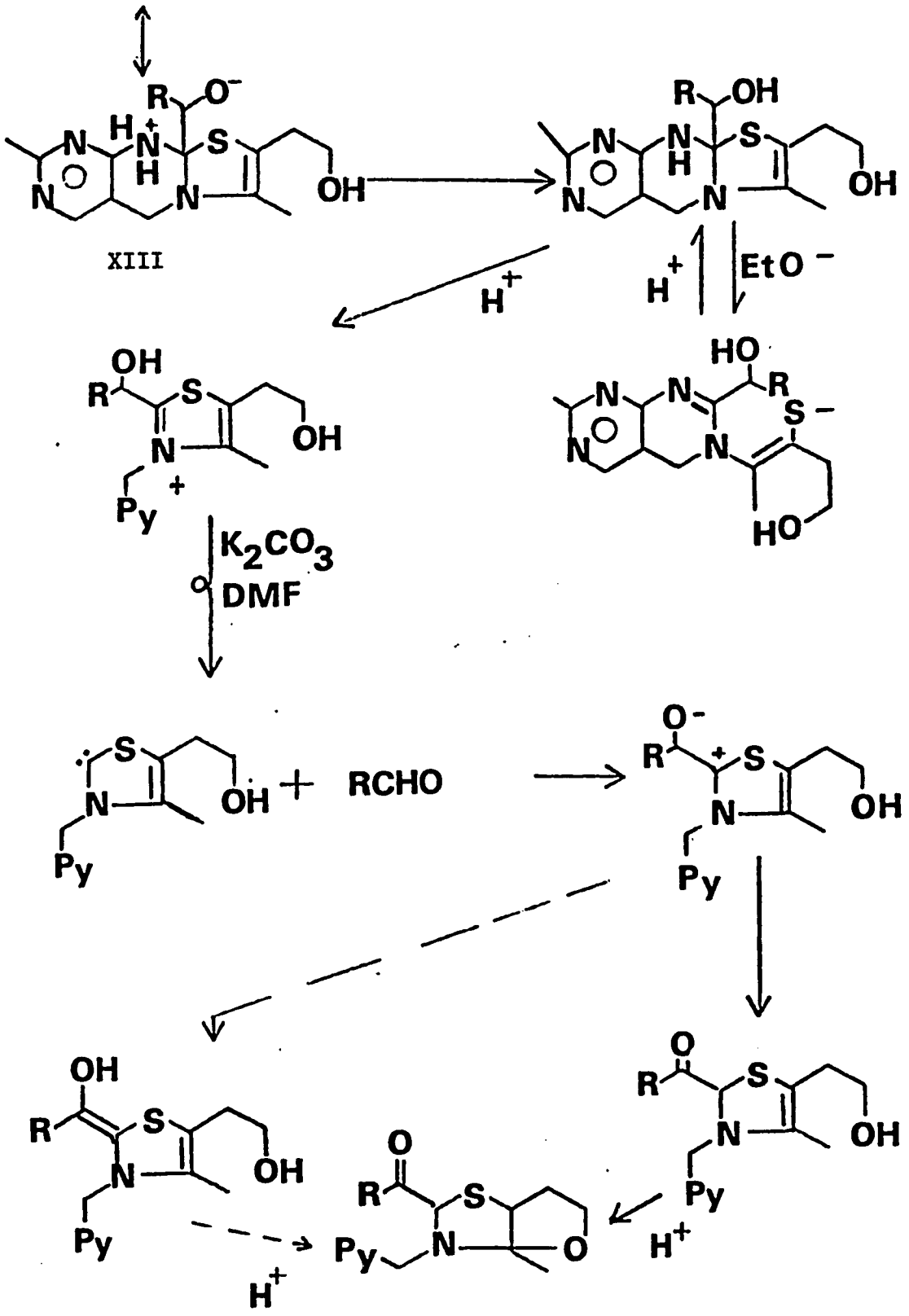



Figure 36 (continued).

proton abstraction generates the anionic intermediate of Metzler's compound which is stabilized by the inductive effects of the hetero atoms. Departure of the least nucleophilic group, the pyrimidyl nitrogen, completes the formation of the carbene (XII).

Reaction of this intermediate with aldehydes results in the formation of the intermediate, (XIII). Under heavily basic conditions used to hydroxyalkylate thiamine (Exp. 1 and 4), the thiazole ring opens up, preventing isomerization to the thermodynamic product, the perhydrofuro ketone. Upon acidification, spontaneous closure of the thiazole ring regenerates its aromatic character and permits the isolation of the hydroxyalkyl thiamine (1 and 3).

When benzaldehyde is used as the alkylated reagent, spontaneous rearrangement of (XIII) produces 5 directly, preventing thiazole ring opening. The cause of this difference is the increased stabilization of aromatic ketones over their aliphatic counterparts. That the ketone isomers can be produced from hydroxyalkyl thiamine compounds[126,203,204] is further proof of the carbene. If the carbene intermediate (XII) does exist, generation of any ketone products should be a result of the carbenoid action of the vitamin.[204] Therefore, to produce HET ketone (3a) from HET (3), the aldehyde moiety must be released and react again to produce the thermodynamic product, 3a. In order to test this hypothesis, HET (Exp. 4) was stirred in DMF with carbonate as a base. Dimedone, an aldehyde specific trapping reagent was added (Exp. 5). Free

acetaldehyde was drained by the dimedone (Fig. 37). It has been suggested[126] that the formation of HET ketone proceeded by simple isomerization. This experiment does not rule out the possibility of isomerization as a competing reaction. However, the very fact that acetaldehyde is released under these conditions demonstrates that the carbenoid mechanism is a viable alternative to Breslow's ylid.

In light of these experimental results, it appears likely that base catalyzed reactions of thiamine proceed through a carbene rather than an ylid, even though they are formally regarded as resonance structures (Fig. 36). Although it is commonly regarded that either heteroatom can participate in the stabilization of the carbene by p orbital overlap with the vacant orbital of the carbene, forming an ylid, nothing has been demonstrated to confirm this. In fact, it has been shown that some carbenoids are not planar, and, thus, there is no resonance overlap of the p orbitals.[205] Furthermore, *ab inito* calculations have demonstrated 70% carbene character to thiazolium salts[186] which do not react with alkyl halides in a manner consistent with ylids. Ylids normally can react with alkyl halides; carbenes cannot. This brings serious doubts to this long standing assumption. Since there is a disparity in the reactivity and since there is no proof that a vinyl ylid is a valid resonance structure for a carbene, the reactions previously discussed are best regarded as operating exclusively through a carbene. The behavior of thiamine is therefore best expressed in terms of its carbene nature because its reactivity is identical to known

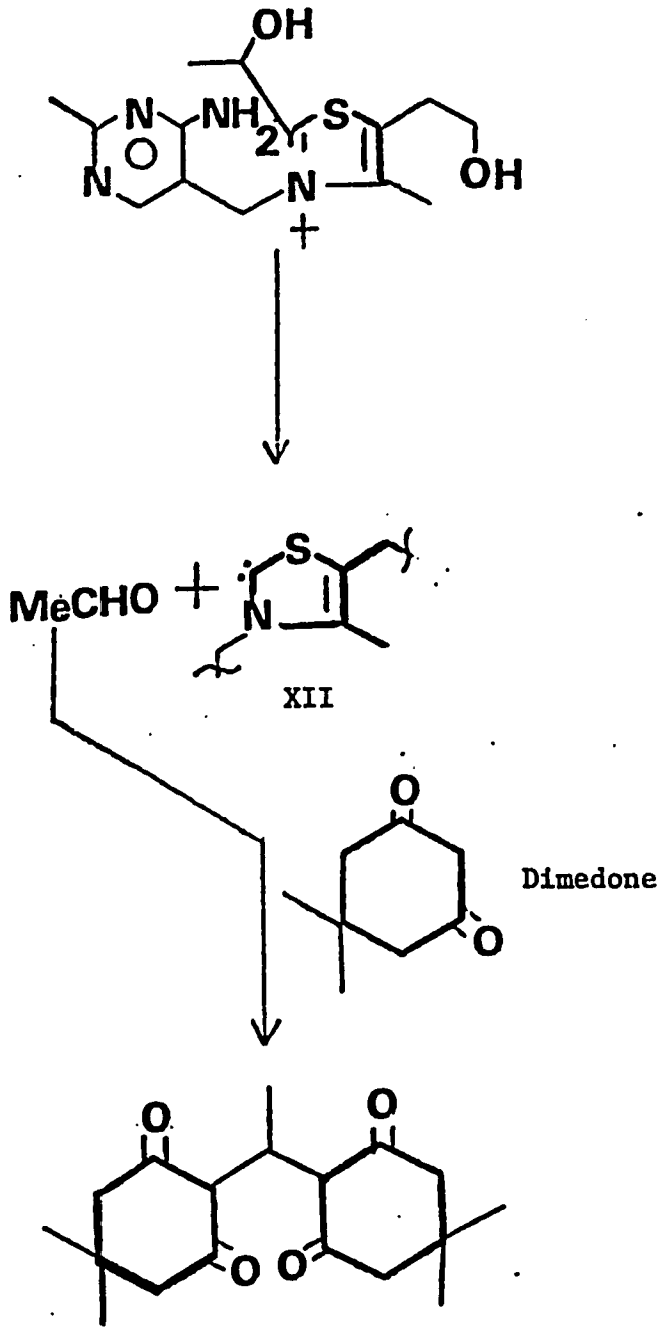



Figure 37. Reaction of HET in the presence of dimedone.

nucleophilic carbenes.[187] It can react with aldehydes but not alkyl halides. An ylid would be expected to react with alkyl halides; thiamine does not.[206] As such, the resonance contribution of the ylid is minimal.

Taking advantage of this carbene nature of thiamine, α,β-unsaturated aldehydes should react in a similar fashion with $B_1$ as benzaldehyde. The same electronic forces that favor spontaneous ketone formation for the phenyl derivative should favor ketone formation for allylic derivatives. Cinnamaldehyde was reacted with thiamine in an identical fashion as performed with benzaldehyde (Exp. 7). No ketone formation was observed and only unreacted thiamine was identified. A variety of mechanistic explanations are proposed (Fig. 38) which illustrate two types of acetoin condensation. The proton ($H_\alpha$) becomes more acidic than in the corresponding saturated analogue, due to the increased stabilization through the olefinic bond. Attack on an unreacted aldehyde may occur at the 1' or 3' position generating thiamine and acetoin products. The only drawback to these schemes is that $H_\alpha$ tends to be acidic regardless of the degree of unsaturation at the adjacent carbons. It is possible that such an anion normally exists only transiently and with α,β-unsaturated aldehyde adducts, a more stable species may be formed. Alternatively protonation of this species would result in the formation of a keto-thiamine compound (XIV). Thiamine is spontaneously released by addition of solvent resulting in the formation of ethyl hydrocinnamate and

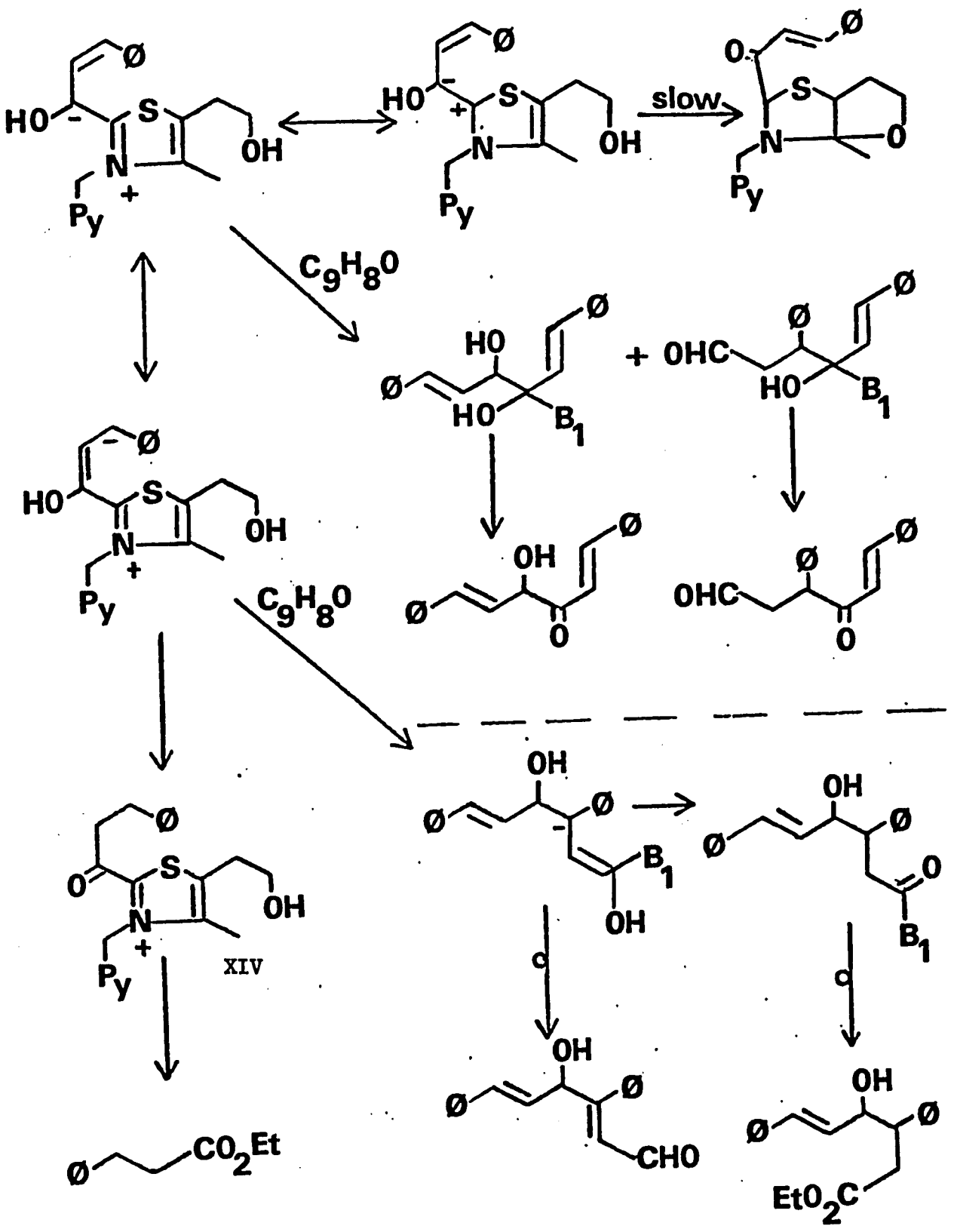



Figure 38. Competing side reactions of thiamine and cinnamaldehyde.

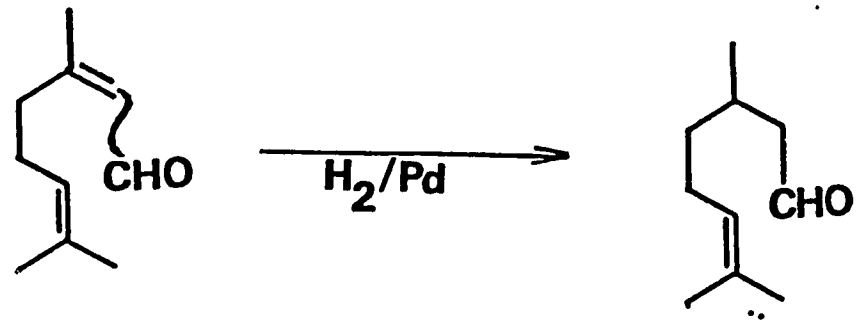
thiamine.[188] Thin layer chromatography of the reaction mixture (Exp. 7) showed a multiplicity of spots, indicating that several side-reactions were occurring simultaneously. As a result of this extraordinary turn of events, synthesis of hydroxyalkyl thiamine compounds utilizing α,β-unsaturated aldehydes was abandonded. Since the saturated counterpart to 2-hydroxygeranyl thiamine is readily obtainable, the effect of this substrate on irregular terpene biosynthesis was more extensively investigated.

Karimian[125,126] had already shown that 2-hydroxycitronellyl thiamine, 1, could serve as an effective biological precursor for diisopentenyldihydroartemisia ketone, 18a, in yeast enzyme preparations. As his experiments utilized only labelled geraniol and unlabelled 1, confirmation of the obligatory role of this thiamine adduct in the biosynthesis of 18a was necessary. The most straightforward method of accomplishing this task was to synthesize $^{3}$H-1 and perform the identical enzymatic experiment as Karimian using this labelled material and cold geraniol. Labelled citronellal, which is necessary for the synthesis of the thiamine compound, must be prepared.

Catalytic hydrogenation should prove to be the most efficient method for selective reduction of $^{3}$H-citral to $^{3}$H-citronellal. Investigations by Adams[138,139] demonstrated that platinum oxide will reduce the conjugated bond of citral preferentially over the isolated olefin. Unfortunately the catalyst is also very effective in reducing the aldehyde to the corresponding alcohol.

By adapting this procedure to palladium catalysts,[207,208] it was possible to eliminate this side reaction. Palladium on carbon (10%) was substituted for platinum oxide (Exp. 3). After absorption of one equivalent of hydrogen, the reaction was terminated. Reduction quantitatively produced citronellal. The preferential hydrogenation at this position occurs for two reasons. First, the carbonyl chelates with the catalyst through its vacant d orbitals, thus allowing the hydrogen to attack the closest olefin. Second, by chelating the carbonyl, the α,β-unsaturated linkage is polarized even more than normal, allowing for a more facile transfer of hydrogens (Fig. 39).

Once the labelled citronellal was obtained, it was easily attached to thiamine (Exp. 1). This compound, $^{3}$H-1, was incubated with unlabelled geraniol in the enzyme preparation (Exp. 30). Chromatography of the extracted terpene fraction, against an authentic sample of 18a,[126] demonstrated that the thiamine compound was, indeed, a precursor to this irregular terpene. The level of incorporation (7%) in this experiment was identical to that found by Karimian when the labelled material was geraniol. To account for this conversion, the following mechanism has been proposed[125,126] (Fig. 40). Thiamine reacts with the aldehyde to form the hydroxyalkyl derivative, 1. $S_N2'$ attack on geranyl pyrophosphate displaces the pyrophosphate and generates the artemisyl adduct. Departure of the thiamine moiety results in the formation of the artemisia ketone analogue, 18a.




citral citronellal

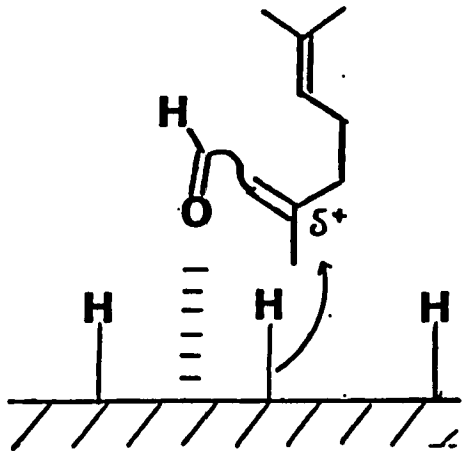
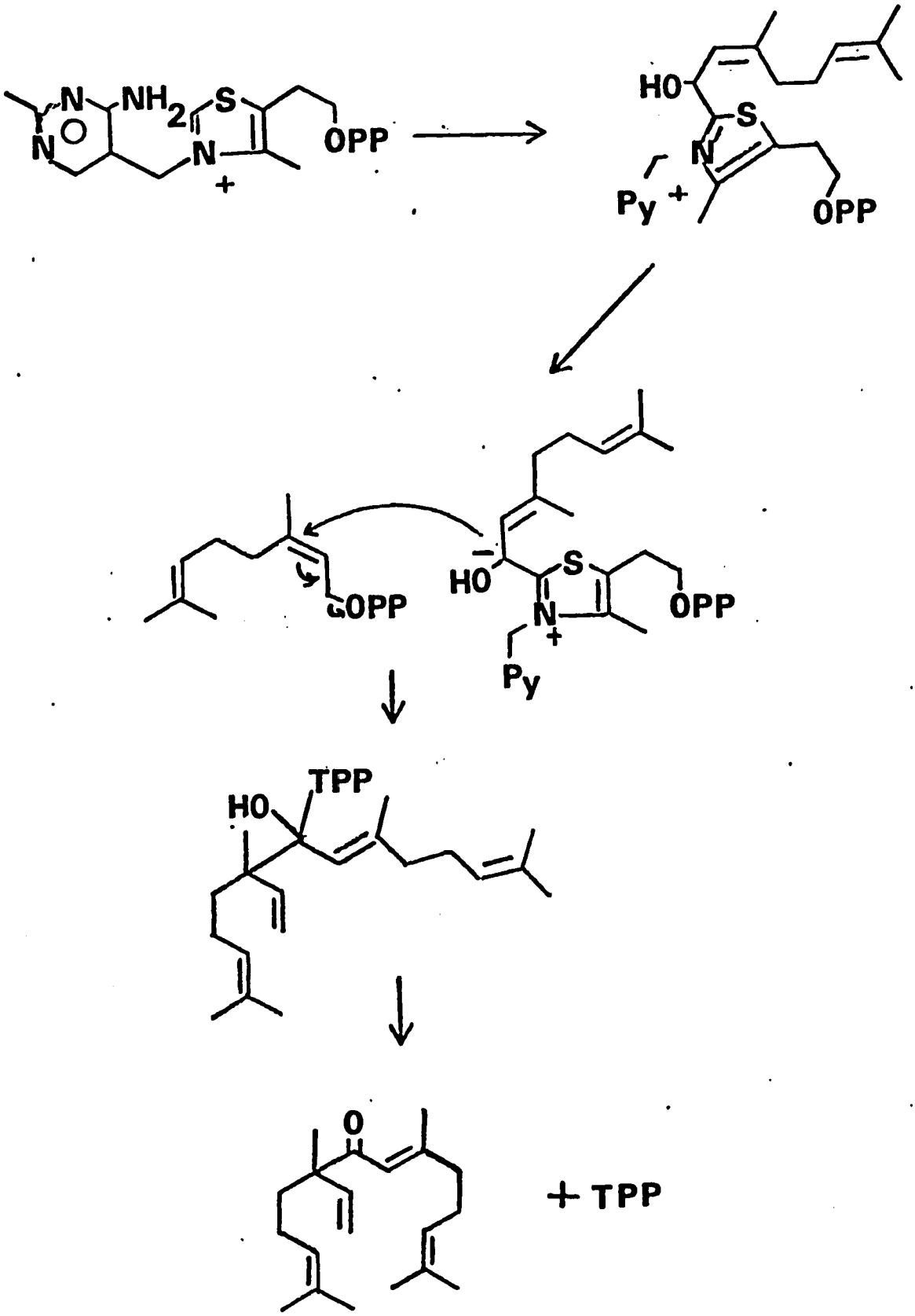



Figure 39. Selective hydrogenation of citral.

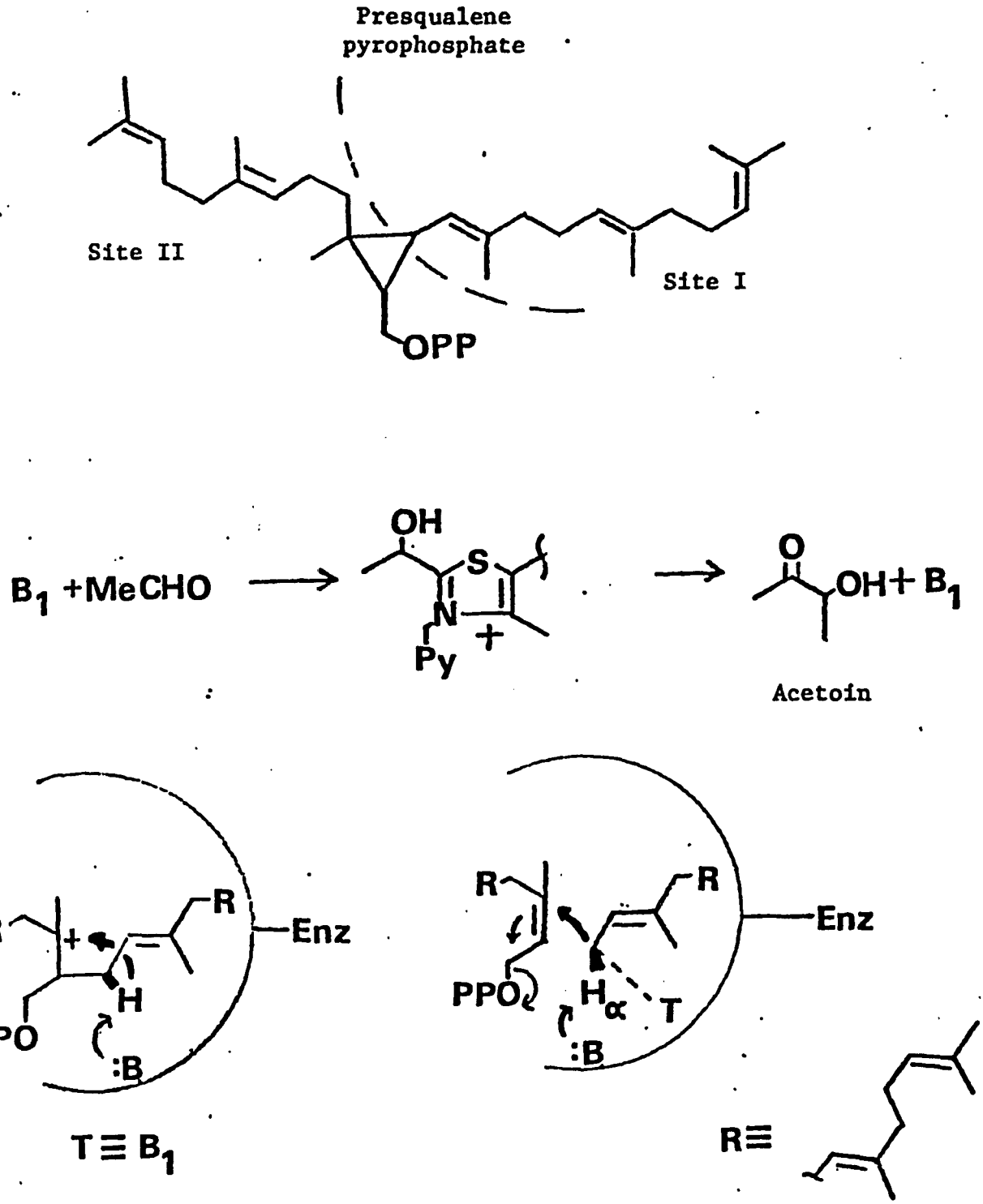



18

Figure 40. Karimian's mechanism for the biosynthesis of 18.

At this point it is important to stress that irregular terpenes of the artemisyl type are not natural products of yeast. Due to the structural similarities of this system with the presqualenyl skeleton (see Figs. 14 and 15), it is probable that squalene synthetase is responsible for this catalysis and the $S_N2'$ attack mimics the 1,3 elimination that forms the cyclopropyl ring in presqualene (Fig. 10). If this is true, then some critical features of the thiamine-terpene adduct, 1, (Fig. 41) must be examined. For instance, the abstraction of the proton ($H_\alpha$) of 1 must either be enzyme catalyzed or mediated by some other mechanism. If the enzyme is responsible then it appears likely that the same enzymatic base which abstracts the proton in squalene synthesis is removing $H_\alpha$ from 1. Unfortunately for this model, the proton removal for the 1,3 elimination occurs on intermediate (XV) which has the two farnesyls covalently linked, whereas two distinct species exist in the reaction involving 1. If the enzyme can hold the two precursors in a rigid configuration, then the necessity of a covalent intermediate such as (XV) is minimal. Since, the enzyme has been found to accept modified substrates[209-211] it is not surprising to find this thiamine-terpene analogue is active. The abstracted proton is, however still adjacent to the departed pyrophosphate.

A more likely explanation for this activity is the nonspecific base abstraction of the acidic proton, $H_\alpha$. It is known that HET can react at physiological pH to form acetoin nonenzymatically[212] (Fig. 41). Therefore, once 1 is bound to the enzyme,




XV

Figure 41. Proposed active site accomodation of a thiamine-terpene analogue and the non-enzymatic formation of acetoin.

catalysis can occur only after the proton is nonenzymatically removed. In order for the thiamine moiety to fit snugly in the active site of the enzyme, it is necessary for at least part of the cofactor to have isoprenoid characteristics. Inspection of molecular models of both the thiamine thiazole and isopentenol reveal some startling similarities. When the 5-hydroxyethyl and the 4-methyl and the sulfur of the thiazole are considered as a whole, this functionality bears a remarkable resemblance to an isoprene, not only with the correct geometry, but with the right degree of unsaturation. The thiazole forces the C-4 methyl and the sulfur into the same plane, thus imitating the two terminal methyls of an isoprene unit.

Drawing upon these observations, it becomes clear that 1 can only act as one specific farnesyl analogue. As a result of the asymmetric reaction of squalene synthetase involving two identical substrates, farnesyl pyrophosphate, it became necessary to characterize each site of the enzyme.[213] Site I is defined as that which binds the first farnesyl moiety. This half of the ultimate squalene molecule loses the proton in the biosynthetic dimerization of the two farnesyl residues.[213,214] Site II binds farnesyl pyrophosphate only after Site I is occupied. This half of the presqualene retains the pyrophosphate. Each site has its own set of binding requirements for both the pyrophosphate and hydrophobic regions of farnesyl pyrophosphate. Since 1 can only act as a Site I substrate, this compound permits the testing of substrate specificity

of Site II substrates. Thus, by substituting farnesol for geraniol in the yeast preparation, a C-25 artemisia ketone analogue, 26, should be biosynthesized (Exp. 31). The standard was chemically synthesized by reaction of farnesyl bromide (Exp. 27) and citronellal on the zinc column (Exp. 28), followed by oxidation with chromium trioxide (Exp. 29). The incorporation level was identical to the amount found for the biosynthesis of 18a (7%). If the low yields of 18a were in part due to the binding of geraniol, an unnatural substrate, to Site II, then the yields should be higher when farnesol was substituted. The failure of any change in the rate of incorporation upon changing the terpene substrate reflects that chain length is not an enzymatic requirement for binding at the second site. Since it has already been demonstrated that pyrophosphate is required for activity by at least one of the binding sites, it stands to reason that this factor is the principal determinant for activity in Site II.

Alternatively, the structural discrepancy between the actual substrate, farnesol, and 1 may be an overriding factor. It may hide any influence the chain length of the Site II substrate exerts upon the enzyme. While it is not possible to assess this directly, it seems likely that such influences whould have a multiplicative effect upon the catalytic activity of the enzyme. Furthermore, considering the flexibility of these sites for binding unnatural substrates[209-211,214-217] such chain length differences should have a minimal effect on the total activity. Clearly the rate limiting

factor present is not the binding of the substrates to the enzyme, but the actual catalytic conversion of 1 and geranyl of farnesyl pyrophosphate to 18a or 29, respectively. It has been demonstrated that Site I is not very specific in its binding of substrates, but very particular about what is actually converted to presqualenyl type compounds. Since a simply modified substrate, such as 4-fluorofarnesyl pyrophosphate cannot react, it should be expected that 1, a radically altered "terpenoid" would be equally unreactive. Its reactivity is most probably due to the acidity of the α-proton. Once the anion is formed the reaction occurs spontaneously. Therefore, the enzyme merely acts as a surface which immobilizes the substrates in the proper conformation to allow the $S_N2'$ reaction to proceed (Fig. 41).

In order to confirm this hypothesis it was necessary to demonstrate that a thiamine-artemisyl derivative could actually be biosynthesized. Only in this way can the formation of these irregular terpenes be unequivocally linked to thiamine or thiamine based compounds. The method for testing this theory would be to isolate an irregular terpene with a thiamine moiety attached. The simplest strategem would be by removal of the pyrimidine ring from 1. Karimian[126] has shown that disubstituted derivatives of thiamine (Fig. 42) spontaneously release under aqueous conditions. Upon removal of the positive charge on the thiazole nitrogen, such release cannot occur. Thus, 23 was sought instead.

The chemical synthesis of the standard was quite simple

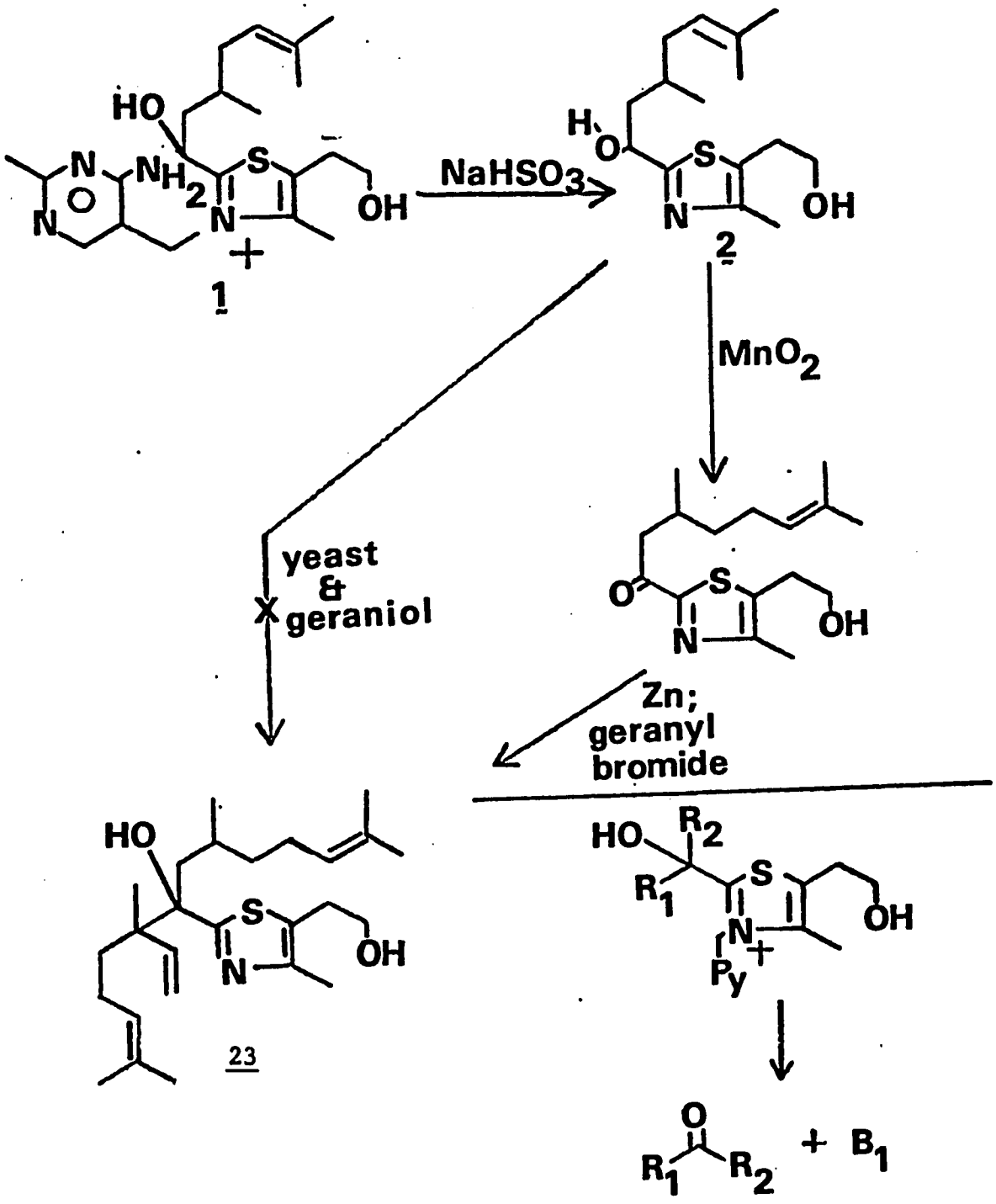



Figure 42. Release of ketones from thiamine and attempted biosynthesis of 23.

and straightforward. Cleavage of the thiamine rings was accomplished by stirring 1 with sodium sulfite (Exp. 2). The resulting hydroxycitronellyl thiazole, 2, served both as a biological precursor and an intermediate in the chemical synthesis of the artemisyl-thiazole derivative, 23. Thus, 2 was oxidized with manganese dioxide (Exp. 25) and alkylated with geranyl bromide via the Reformatsky reaction (Exp. 26) to generate the artemisyl derivative, 23. Structural conformation was determined by NMR (NMR 19). The presence of the angular methyl (δ 1.1), the thiazole methyl (δ 2.5), and the AA'B vinyl splitting downfield (δ 4.8-5.8) as well as the two triplets signifying the 5-hydroxyethyl moiety of the thiazole, all demonstrate the validity of the proposed structure.

Geraniol and 2 were incubated in the cell-free enzyme system (Exp. 32) in order to test the validity of the hypothesis. Alkaline phosphatase was added to remove any pyrophosphate covalently linked to the hydroxyethyl moiety. Upon extraction and chromatography, none of the desired compound was obtained. The most probable explanation for this is the inability of the α-proton of 2 to solvolyze or to be easily abstracted. Chemical models bear this out (Fig 43). In the chemical synthesis of 2-alkylthiazoles other activation (e.g., a carboethoxy group) is required. Thus, the thiazole itself is not capable of supporting an anionic species.[218] Furthermore, the proton on the 2-methylthiazole has been shown to resist abstraction with n-butyllithium.[219] In comparing this system with

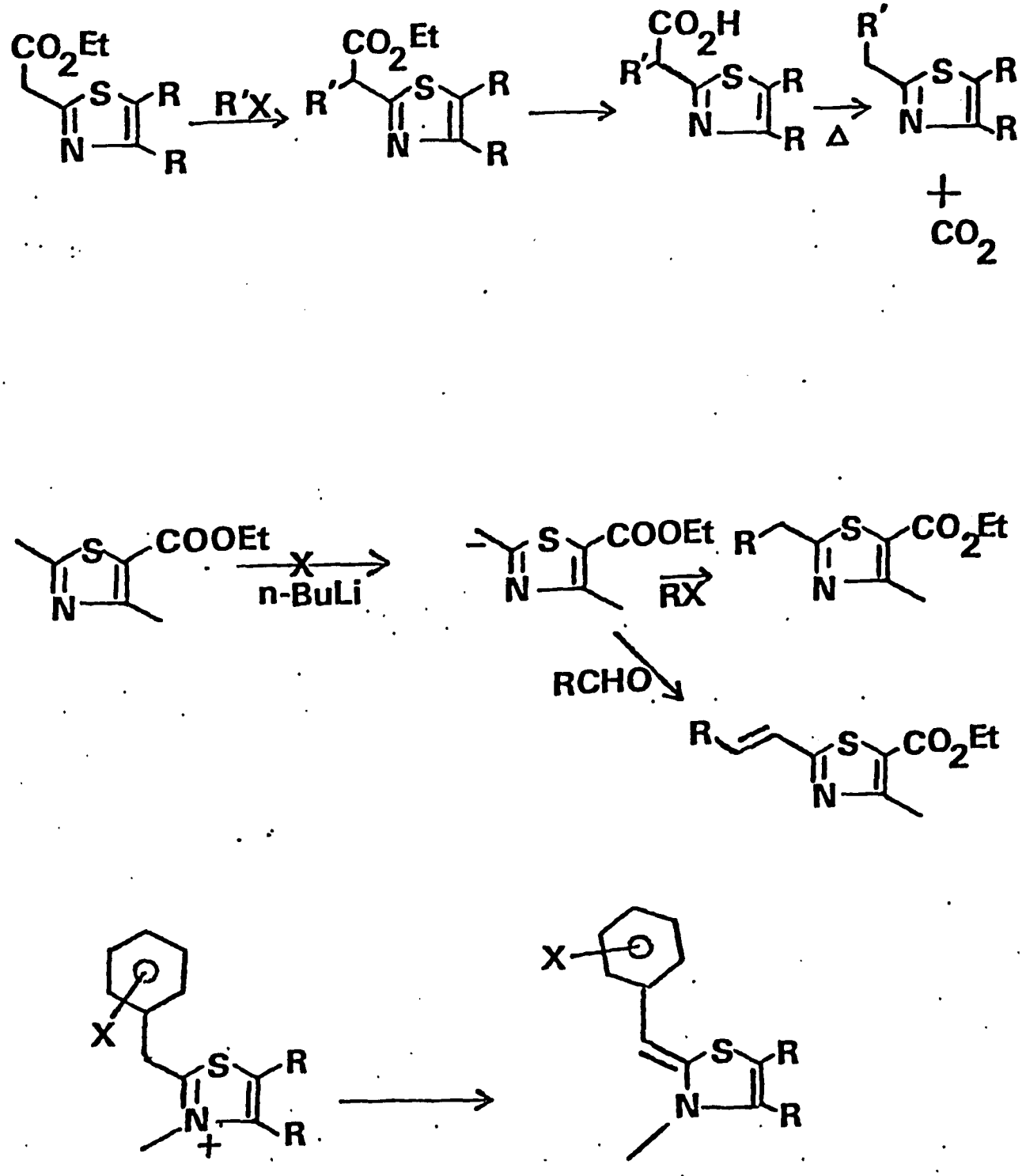



Figure 43. Reactions of thiazoles.

the corresponding N-alkyl thiamine, two major differences can be found. First, developing a negative charge on the α-methyl of the thiazole disrupts the aromaticity of the thiazole ring. When the same is done on the thiazolium salt, this disruption is compensated by removal of the positive charge on the ring. Second, the enamine formed is a stable discrete intermediate.[220] In forming this species, the N-substituent can assume a trans configuration in relation to the C-4 moiety, thus creating a more stable system.

Because a positive nitrogen is necessary to stabilize any enamine formed from a thiamine or thiazole adduct, it was decided that 2-alkyl derivatives of thiamine should be investigated. These compounds would have no α-hydroxyl group but still retain the positive charge on the thiazole nitrogen. In this way a thiamine-terpene compound could not release the C-2 side chain. Furthermore, these compounds would have more substrate characteristics as farnesol does not normally have any internal hydroxy groups. The strategy for syntheses of these thiamine derivatives was by no means simple. The thiamine moiety has been demonstrated to be especially base sensitive[126] and the terpene is, of course, acid labile. It was, therefore necessary to utilize mild conditions whenever possible.

Several schemes were developed to accomodate this problem (Fig. 44). Scheme I represents an approach taken by Karimian [126] Since 1 proved too labile for conventional modifications, the cleavage product, 2, was investigated. It was found that the α-carbon could be reduced to the methylene. Unfortunately the thiazole

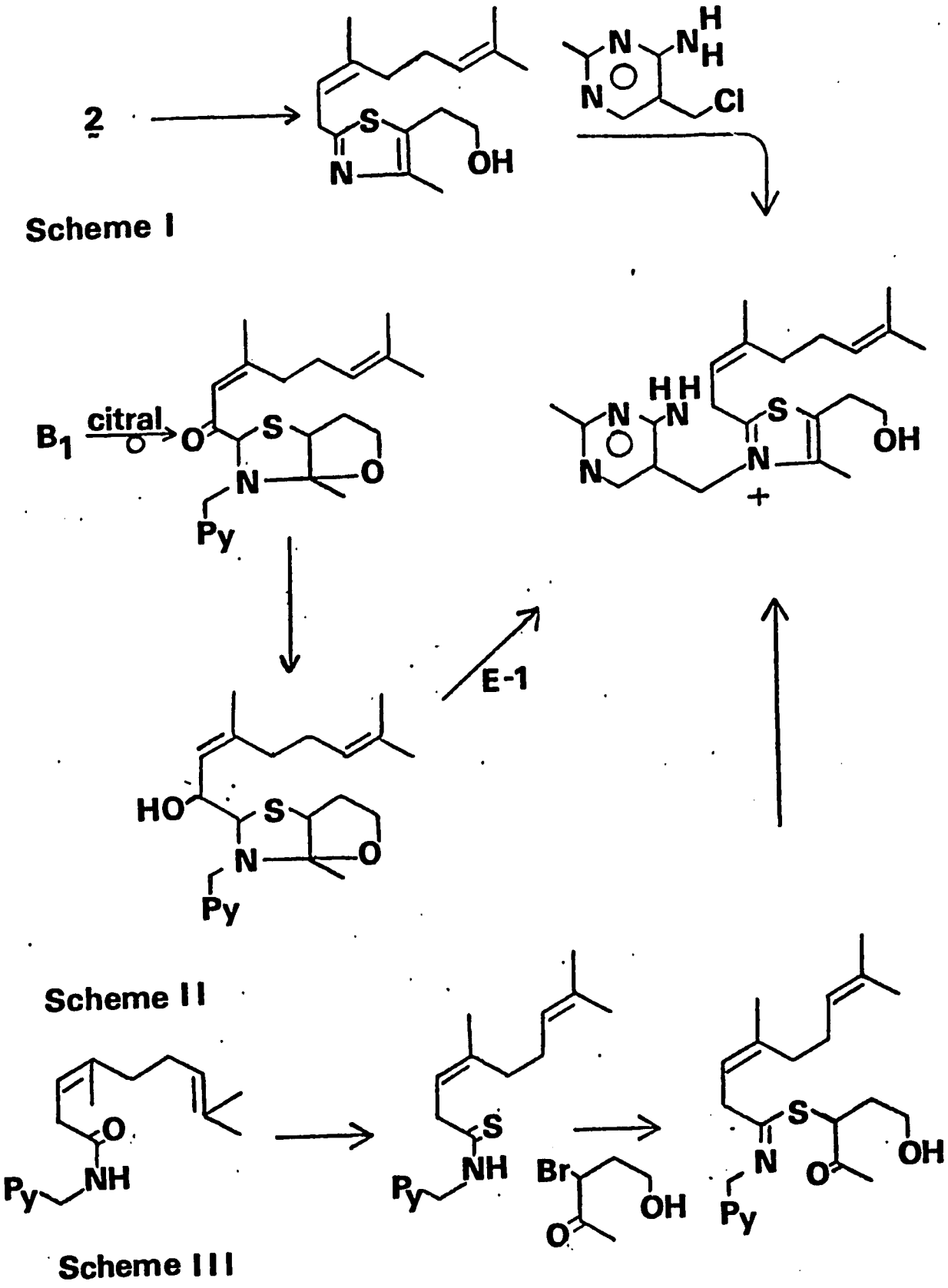



Figure 44. Synthetic schemes for 2-geranyl thiamine.

nitrogen could not be realkylated. The cause of this inability lies with the steric hindrance produced by the terpene at C-2 and the methyl at C-4. These groups prevent the pyrimidyl chloride from approaching the nitrogen.

Alternatively, Scheme II was then investigated. The formation of perhydrofuro derivatives were potentially more resistant to decomposition than the parent thiazole because there is no charged species. Also, the 2, 3, and 4 substituents can assume a trans orientation and thus, relieve the steric compression brought about by the planar configuration of the thiazole. But, the starting aldehyde must contain an α,β-unsaturated linkage in order to undergo mild E-1 elimination once it is bound to thiamine. Therefore, this method was abandoned along with the synthetic scheme for 2-hydroxygeranyl thiamine.

Attention was then shifted to a method employing the Hantzsch Thiazole Synthesis (Scheme III).[221] To this end, the following synthetic approach was taken (Fig. 45). The first model compound targeted for synthesis was 2,3,4-triphenylthiazolium chloride, 12. The availability of starting material made the investigation of this compound ideal. Also, if the steric factors, produced by the phenyl groups lying in the same plane, could be overcome, synthesis of 2-geranyl thiamine should present no problem. Although synthesis of thiobenzanilide (Exps. 11 and 12) proved to be no problem, S-alkylation was found to be a difficult procedure. It was found that once base was added to the mixture the

reaction proceeded smoothly (Exp. 12). At first it was believed that the base was necessary to make the thioamide reactive (by formation of the thiolate anion) because the thioamide was intrinsically unreactive. However, other factors soon presented themselves which explained this apparent unreactivity (see below).

With the S-phenacyl thiobenzanilide (11) in hand, attempts to close it to the thiazolium salt were undertaken. Thionyl chloride was chosen as the dehydrating reagent. Upon addition of this reagent to 11, dissolved in benzene (Exp. 13), none of the desired material was produced. Apparently the steric factors were too great to allow ring closure. In the transition state as well as the final form, the three phenyl groups must lie coplanarly with the forming thiazole (Fig. 45). Construction of molecular models will demonstrate the strain involved in ring closure. In an attempt to circumvent this problem, a potentially less bulky octyl group was chosen to replace the phenyl at C-2. In this way the steric factors of this moiety would mimic the geranyl functionality of 2-geranyl thiamine. Thus, nonanilide (Exp. 14) was subjected to phosphorus pentasulfide in pyridine (Exp. 15). The resulting tar resisted all attempts to recrystallization. A convenient method was developed that purified this low melting solid. The solution was passed through a bed of alumina which trapped all of the undesirable side products on the column. The relatively pure thioamide, 14, was eluted and recrystallized with ease.

When alkylation with any phenacyl halide was attempted under

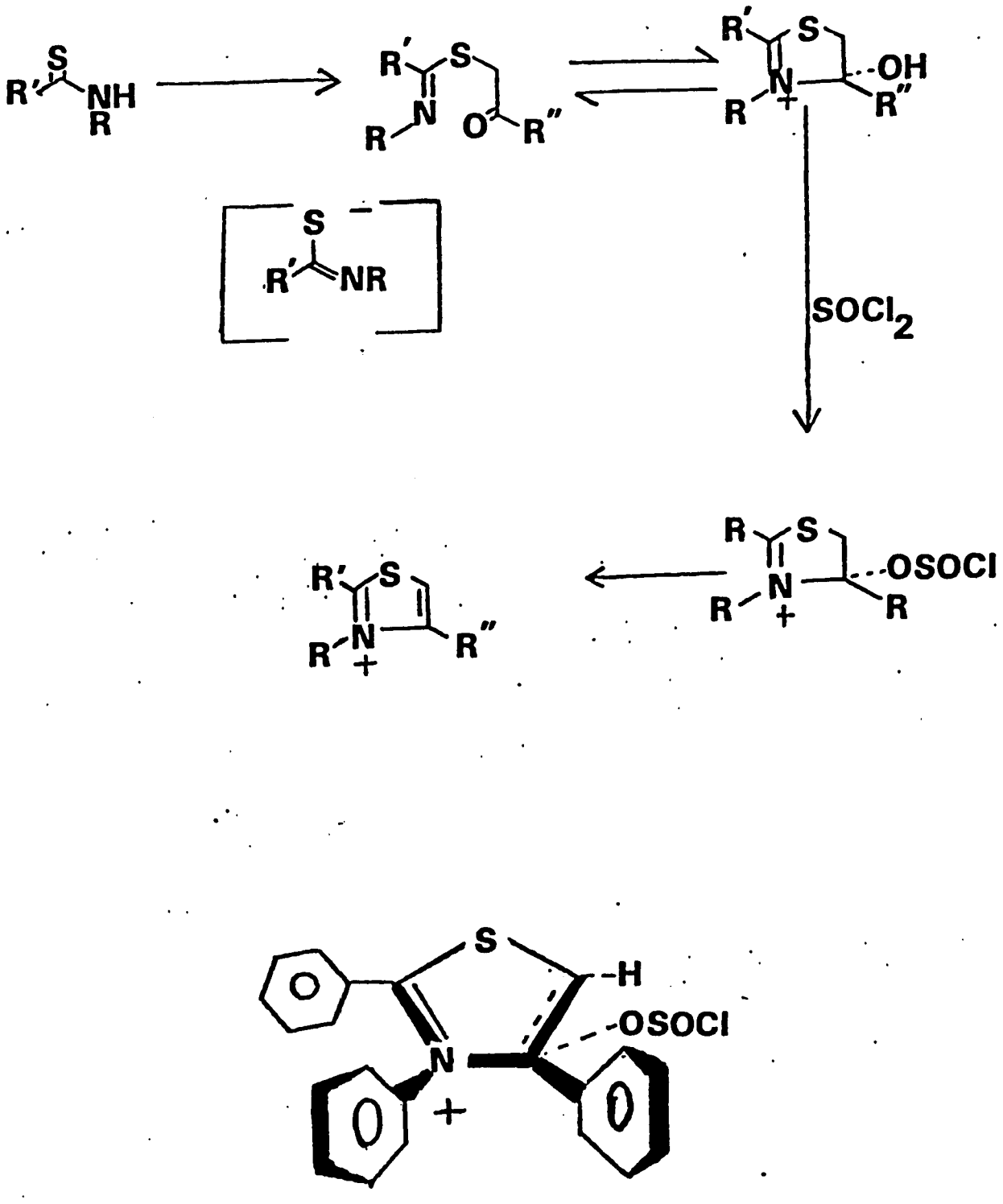



Figure 45. Model systems in the Hantzsch Thiazole Synthesis

base conditions, none of the S-alkylated material was obtained. After many frustrating failures, it was found that α-keto bromides and the thioamide in the absence of base could easily accomplish the desired synthesis. As such *p*-chlorophenacylbromide and 14 were reacted together in methylene chloride. Very quickly the S-alkyl derivative, 15, was obtained (Exp. 16). Unlike thiobenzanilide, thiononanilide could undergo side reactions in the presence of base through the abstraction of the α-proton. To compensate for the removal of the base, a phenacyl bromide was substituted for phenacyl chloride. Since this reaction (Exp. 16) proceeded smoothly, it appears that the rate limiting factor was not the thioamide, but the relative efficiency of the particular halide to act as a leaving group. Comparison of the spectra of 11 and 15 produced some interesting conclusions. Compound 11 shows a carbonyl absorption at 5.9 microns (IR 4) and a downfield resonance at δ 6.5 (NMR 9) for the phenacyl methylene. This leads one to believe that 11, the free base, exists in an open form. The spectra of 15, however, do not fit with the same conclusion. The lack of a carbonyl absorbance and downfield resonance (IR 7 and NMR 12, respectively) reveal that 15, the hydrobromide salt of the S-alkyl derivative of this nonanilide, exists as the 4-hydroxythiazoline. Based on the NMR and IR spectra, it is clear that the thiazoline zwitterion is not an appreciable resonance structure of 11 because the preferred conformation is produced by rotating the large substituents about the sulfur, away from each other.

On the other hand, since 15 exists as the hydroxythiazoline, it was felt that simple dehydration would result in the formation of the desired thiazole, 16. As such, 15 was reacted with thionyl chloride in benzene (Exp. 17). The material turned purple, but no product was isolated and TLC showed only starting material present. Again, steric strain seems to be the overriding factor governing the fate of the reaction. Although Egan[222] successfully formed thiazolium salts from the corresponding 4-hydroxythiazolines, the substituents were small enough to accomodate a planar arrangement. The substituents on 11 and 15 are not.

With the methods for the synthesis of 2-geranyl thiamine exhausted, assessment of the current status of the work and future research is in order. An alternative synthetic method for this compound may be achieved by utilizing 4,5-thiazolines (Fig. 46). These compounds could be synthesized by reacting α-mercaptoketones with Schiff's Bases.[223,224] The resulting thiazoline would be stable because the 2 and 3 substituents could assume a trans configuration. Upon the synthesis of the thiamine analogue, the 4,5 thiazoline would form the perhydrofurothiazole, characteristic of reduced thiamine compounds. Two methods may be employed for the completion of the synthesis of 2-geranyl thiamine. If the starting aldehyde was the dehydro terpene, then acid isomerization would generate the desired product. On the other hand, if the saturated analogue were employed, then generation of the thiamine thiazole could be achieved by oxidation with iodine.[126]

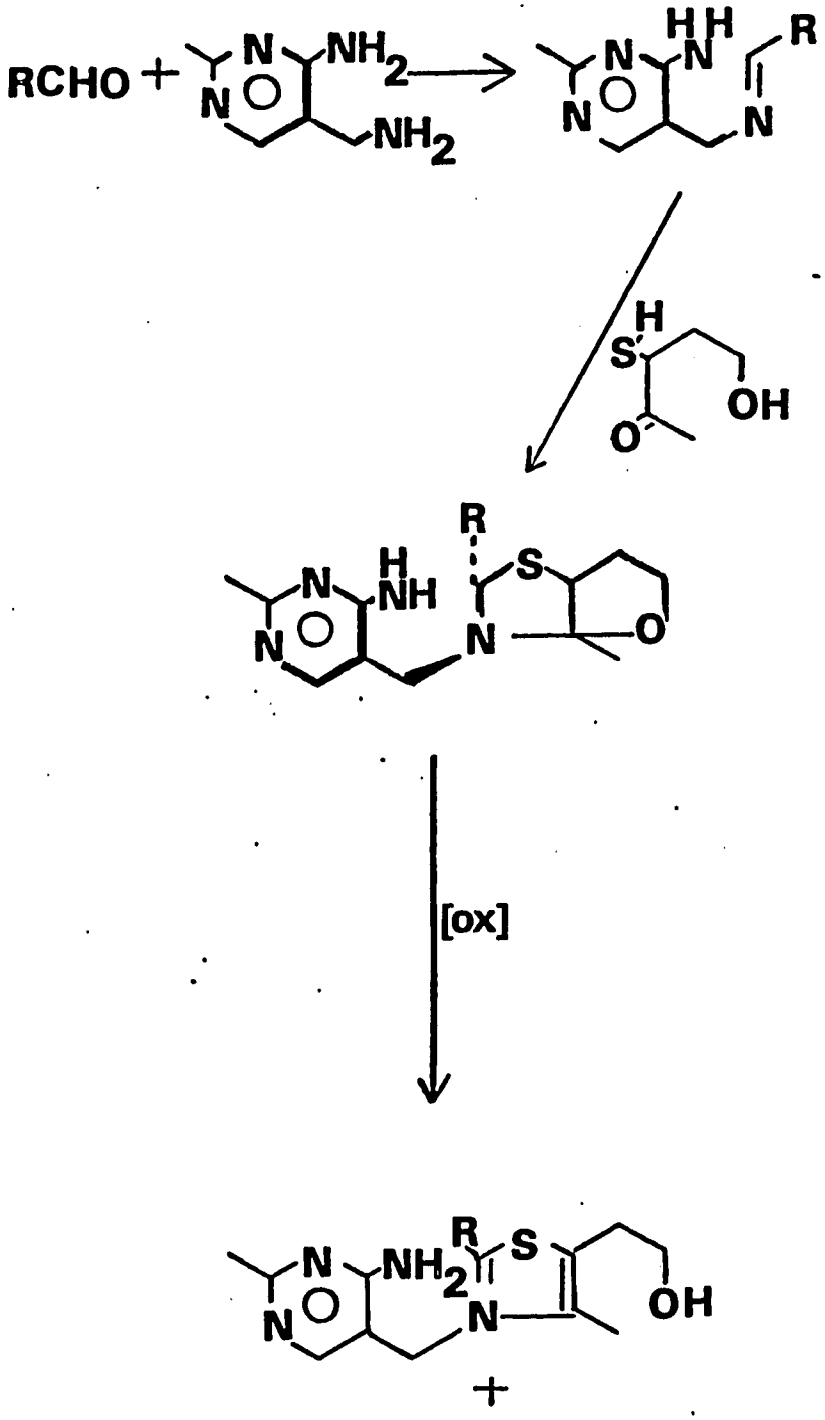



Figure 46. An alternative method for synthesis of alkyl thiamine compounds.

However, in view of previous failures with complex thiamine-terpene compounds, a more fruitful approach to the study of these compounds on terpenoid biosynthesis is now being undertaken. Since the thiazole has many characteristics similar to an isoprene, a study of the effect of a variety of terpenoid thiazoles on cholesterol biosynthesis should result in a new class of competitive inhibitors. As these compounds could be constructed in a variety of ways the prospect of success is much greater. Unlike their thiamine counterparts, the thiazoles could be subjected to harsher conditions as the sensitive pyrimidine group is not present.

In a nutshell, the isolation and characterization by chemical synthesis of an irregular terpenoid with the structure of diisopentenylartemisia ketone, 18, has prompted investigations that have linked thiamine to the biosynthesis of this irregular terpene. Confirmation of this tenet was achieved when 2-hydroxycitronellyl thiamine, 1, was incorporated into a dihydro analogue, 18a, by yeast enzymes. Thus, by extension to regular biological systems, it is proposed that artemisia ketone (I) is biosynthesized via the thiamine adduct, 2-hydroxyisovaleryl thiamine (Fig. 21). Similarly, bakuchiol (II) is biosynthesized from 2-hydroxy-(*p*-hydroxyphenyl)-ethyl thiamine, a tyrosine metabolite, and geranyl pyrophosphate (Fig. 22). In both cases $S_N2'$ attack of the thiamine adduct on the terpene (DMAPP and geranyl pyrophosphate, respectively) produces compounds which can easily be converted to artemisia ketone and bakuchiol, respectively.

In our attempts to ascertain the exact mechanism of thiamine in the biosynthesis of irregular terpenes, attempts to synthesize 2-hydroxygeranyl thiamine were undertaken. Although the targeted compound was not made, the investigations have let to a reevaluation of the mechanism of thiamine. Thus, it appears that thiamine reacts with aldehydes via a carbene, rather than the ylid proposed by Breslow.[188,189]

Woodward's[128,129] suggestion that thiamine is involved in squalene biosynthesis is probably incorrect. Although thiamine-terpene compounds were shown to be active, it appears that this is a result of the structural similarities of the thiamine thiazole pyrophosphate with an isopentenyl pyrophosphate. Consequently, the binding of 2-hydroxycitronellyl thiamine pyrophosphate to squalene synthetase occurs as a result of the similarity of this compound with farnesyl pyrophosphate.

In final retrospect, the lack of any concrete knowledge on either these plant systems or squalene synthetase demonstrates the impossibility of ruling out any of the proposed mechanisms, whether it be Woodward's (Fig. 24) or any other. Furthermore, the relative complexity of these systems make most experimental strategies inherently ambiguous, so that interpretation must be done most carefully. It is these problems that make the study of irregular terpenes both challenging and frustrating at the same time. Also, it is these problems that will make this field controversial for some time.

## REFERENCES


1. D. V. Banthorpe and B. V. Charlwood in "Chemistry of Terpenes and Terpenoids," A. A. Newman, ed. Academic Press, London (1972).

2. M. P. E. Berthelot, "Chemie Organique Fondée sur la Synthese," Millett-Bachelier (1860) p. 733.

3. J. Williams, Jahresber., 495 (1860).

4. E. Halsam, ed, "Comprehensive Organic Chemistry," Vol. 6, Pergamon Press, Oxford (1979) p. 984.

5. C. Harris and A. Himmelmann, Ber. 41, 2921 (1908).

6. W. A. Tilden and W. A. Shenstone, J. Chem. Soc., 554 (1877).

7. A. O. Wallach, J. Chem. Soc., 1582 (1932).

8. O. Jacobson, Ann., 157, 232 (1871).

9. F. Tiemann, Ber., 31, 3278 (1898).

10. Haarman and Riemer, Patentschrift des Deutschen Reiches, 149, 603 (1898).

11. L. F. Fieser and M. Fieser, "Organic Chemistry," 3rd ed., D. C. Heath and Co., Boston (1957) p. 936.

12. Schimmel and Co., Berichte vom April, 14 (1898).

13. A. O. Wallach, "Terpenes and Camphor," 2nd ed., Vit and Co., Lipzig (1914).

14. L. Ruzicka and J. Meyer, Helv. Chim. Acta, 4, 505 (1921).

15. L. Ruzicka, Proc. Chem. Soc., 341 (1959).

16. L. Ruzicka, Experientia, 9, 357 (1953).

17. L. Ruzicka, A. Eschemoser, O. Jeger, and D. Arigoni, Helv. Chim. Acta, 38, 1890 (1955).

18. M. Tsujimoto, J. Chem. Ind. (Japan), 19, 277 (1906).

19. I. M. Heilbron, E. D. Kamm, and W. M. Owens, J. Chem. Soc., 1630 (1926).

20. P. Karrer and A. Helfenstein, Helv. Chim. Acta, 14, 78 (1931).

21. H. J. Channon, Biochem. J., 20, 400 (1926).

22. R. Sonderhoff and H. Thomas, Ann., 530, 195 (1937).

23. K. Bloch, L. C. Clark, and I. Haray, J. Biol. Chem., 211, 687 (1954).

24. K. Bloch, "The Harvey Lectures, 1952/53," 48, 68 (1953).

25. J. W. Cornforth, G. D. Hunter, and G. Popják, Biochem. J., 54, 597 (1952).

26. R. G. Langdon and K. Bloch, J. Biol. Chem., 200, 129 (1953).

27. R. G. Langdon and K. Bloch, Ibid., 135.

28. P. A. Tavormina, M. H. Gibbs, and J. W. Huff, J. Am. Chem. Soc., 78, 4498 (1956).

29. P. A. Tavormina, M. H. Gibbs, J. Am. Chem. Soc., 79, 2646 (1957).

30. H. R. Skeggs, L. D. Wright, E. L. Cresson, C. D. E. MacRae, C. H. Hoffman, D. E. Wolf, and K. Folks, J. Bacterol., 72, 519 (1958).

31. H. R. Skeggs, L. D. Wright, E. C. Cresson, C. D. E. MacRae, C. H. Hoffman, D. E. Wolf, and K. Folks, J. Am. Chem. Soc., 78, 5263 (1956).

32. D. E. Wolf, C. H. Hoffman, P. E. Aldrich, H. R. Skeggs, C. D. Wright, and K. Folks, J. Am. Chem. Soc., 78, 4499 (1956).

33. D. E. Wolf, C. H. Hoffman, P. E. Aldrich, H. R. Skeggs, C. D. Wright, and K. Folks, J. Am. Chem. Soc., 79, 1486 (1957).

34. F. Lynen and V. Henning, Angew. Chem., 72, 820 (1960).

35. S. Chaykin, J. Law, A. H. Phillips, T. Titchen, and K. Bloch, Proc. Nat. Acad. Sci., USA, 44, 948 (1958).

36. V. Henning, I. Kessel, and F. Lynen, "Abstr. Intern. Cong. Biochem., 4th Congr., Vienna," 47 (1953).

37. F. Lynen, H. Eggerer, V. Henning, and K. Kessel, Angew. Chem., 70, 738 (1958).

38. E. Redfearn and J. Glover, Biochem. J., 54, viii (1953).

39. B. W. Agranoff, H. Eggerer, V. Henning, and F. Lynen, J. Am. Chem. Soc., 81, 1254 (1959).

40. D. V. Banthorpe, B. V. Charlwood, and M. J. O. Francis, Chem. Rev., 72, 115 (1972).

41. E. Wenkert, Chem. and Ind. (London), 282 (1955).

42. E. Wenkert and J. Chamberlin, J. Am. Chem. Soc., 81, 688 (1959).

43. A. R. Battersby, Pure and Appl. Chem., 14, 117 (1967).

44. Y. Takeda and H. Inouye, Chem. Pharm. Bull., 24, 79 (1976).

45. H. J. Channon and G. R. Tristram, Biochem. J., 31, 738 (1937).

46. R. Robinson, J. Soc. Chem. Ind. (London), 53, 1062 (1934).

47. F. Lynen, B. W. Agranoff, H. Henning, and E. M. Moslein, Angew. Chem., 70, 657 (1958).

48. H. C. Rilling, J. Biol. Chem., 241, 3233 (1966).

49. E. J. Corey and P. Ortiz de Montellano, Tet. Lett., 5113 (1968).

50. H. C. Rilling and W. W. Epstein, J. Am. Chem. Soc., 91, 1042 (1969).

51. W. W. Epstein and H. C. Rilling, J. Biol. Chem., 247, 4597 (1960).

52. L. J. Aultman, R. C. Kowerski, and H. C. Rilling, J. Am. Chem. Soc., 93, 1782 (1971).

53. J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popják, Proc. Roy. Soc. (London), Ser. B, 163, 492 (1966).

54. G. Popják, G. J. Schroepfer Jr., and J. W. Cornforth, Biochem. Biophys. Res. Commun., 6, 438 (1962).

55. J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, G. Rayback, and G. J. Schroepfer Jr., Proc. Roy. Soc. (London), Ser. B., 1963, 436 (1966).

56. J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, G. Rayback, and G. J. Schroepfer Jr., Biochem. Biophys. Res. Commun., 11, 129 (1963).

57. C. Donninger and G. Popják, Proc. Roy. Soc. (London), Ser. B, 163, 465 (1960).

58. J. E. Edmond, G. Popják, S. M. Wang, and V. P. Williams, J. Biol. Chem., 246, 6254 (1971).

59. H. C. Rilling, C. D. Poulter, W. W. Epstein, and B. Larson, J. Am. Chem. Soc., 93, 1783 (1971).

60. E. E. van Tamelen and M. A. Schwartz, J. Am. Chem. Soc., 93, 1780 (1971).

61. B. H. Davies and R. F. Taylor, Pure and Appl. Chem., 47, 211 (1976).

62. G. Britton in "Chemistry and Biochemistry of Plant Pigments," 2nd ed., T. W. Goodwin, ed., Academic Press, London (1976), Vol. 1, p. 262.

63. D. E. Geronis and H. C. Rilling, Biochemistry, 13, 1538 (1974).

64. G. Britton, Pure and Appl. Chem., 47, 223 (1976).

65. R. B. Bates and S. K. Paknikar, Tet. Lett., 1453 (1965).

66. D. V. Banthorpe and B. V. Charlwood, Nature (New Biology), 232, 285 (1971).

67. G. Pattenden, C. R. Popplestone, and R. Storer, J. C. S. Chem. Commun., 290 (1975).

68. W. W. Epstein and C. D. Poulter, Phytochemistry, 12, 737 (1973).

69. K. Alexander and W. W. Epstein, J. Org. Chem., 40, 2576 (1975).

70. S. A. AbouDonia, C. F. Doherty, and G. Pattenden, Tet. Lett., 3477 (1973).

71. R. Grandk, W. Messerotti, and V. M. Pagnoni, Phytochemistry, 15, 1770 (1976).

72. A. J. Falk, L. Bauer, C. L. Belk, and S. J. Smolenski, Lloydia, 37, 598 (1974).

73. J. P. Haligan, Ecology, 56, 999 (1975).

74. M. M. Yazawa and H. Kameoka, Phytochemistry, 16, 1054 (1977).

75. M. Hurabielle, R. Tilleguin, and T. Noble, Planta Med., 31,

76. T. A. Noble and W. W. Epstein, Tet. Lett., 3931 (1977).

77. T. A. Noble and W. W. Epstein, Ibid., p. 3933.

78. S. K. Paknikar and J. Veeravilla, Indian J. Chem., Sect. B, 183, 269 (1979).

79. J. Shaw, W. W. Epstein, and T. Noble, J. C. S. Chem. Commun., 590 (1975).

80. E. H. Hoeger, Proc. Montana Acad. Sci., 33 (1973).

81. B. M. Trost and R. LaRochelle, Tet. Lett., 3327 (1968).

82. R. Robinson, "Structural Relations of Natural Products," Clarden Press, Oxford (1955), p. 14.

83. G. R. Clemo, Perf. Essen. Oil Rec., 41, 435 (1950).

84. L. Ruzicka, Pure Appl. Chem., 6, 493 (1963).

85. R. B. Bates and D. Feld, Tet. Lett., 4875 (1967).

86. C. D. Poulter, L. L. March, J. M. Hughes, J. C. Argyle, D. M. Satterwhite, R. J. Goodfellow, and S. G. Moesinger, J. Am. Chem. Soc., 99, 3816 (1977).

87. G. Pattenden and R. Storer, Tet. Lett., 3973 (1973).

88. D. V. Banthorpe, S. Doonan, and J. A. Gutowski, Phytochemistry, 16, 85 (1977).

89. K. G. Allen, D. V. Banthorpe, B. V. Charlwood, and C. M. Voller, Phytochemistry, 16, 79 (1977).

90. T. Suga, T. Shishibori, K. Kotera, and R. Fujii, Chem. Lett., 533 (1972).

91. D. V. Banthorpe, B. V. Charlwood, H. M. Greaves, and C. M. Voller, Phytochemistry, 16, 1387 (1977).

92. M. P. Crowley, P. J. Godin, H. S. Inglis, M. Snarey, and E. M. Thain, Biochem. Biophys. Acta, 60, 312 (1962).

93. D. V. Banthorpe and B. V. Charlwood in "Terpenoids and Steroids," J. R. Hanson, ed., The Chemical Society, Burlington House, London (1977), Vol. 7, p. 176.

94. W. D. Loomis and R. Croteau in "Recent Advances in Phytochemistry," V. C. Runeckles and T. J. Mabry, eds., Academic Press, New York (1973), Vol. 6, p. 147.

95. T. Suga, T. Hirata, T. Shishibori, and K. Tange, Chem. Lett., 243 (1975).

96. T. Suga, Chem. Lett., 131 (1975).

97. T. Suga, Ibid., p. 243.

98. D. V. Banthorpe, J. Mann, and K. W. Turnbull, J. Chem. Soc. (C), 2689 (1970).

99. D. V. Banthorpe and D. Baxendale, J. Chem. Soc. (C), 2694 (1970).

100. K. G. Allen, D. V. Banthorpe, B. V. Charlwood, D. Ekundayo, and J. Mann, Phytochemistry, 15, 101 (1976).

101. T. Suga and T. Shishibori, Bull. Chem. Soc. Japan, 46, 3545 (1973).

102. D. V. Banthorpe, G. A. Bucknall, H. J. Doonan, S. Doonan, and M. G. Rowan, Phytochemistry, 15, 91 (1976).

103. M. J. O. Francis, D. V. Banthorpe, and G. N. Patoorel, Nature, 228, 1005 (1970).

104. T. Suga, Phytochemistry, 10, 2725 (1971).

105. E. Lanza and J. K. Palmer, Phytochemistry, 16, 1555 (1977).

106. T. Wuu and D. J. Baisted, Phytochemistry, 12, 1291 (1973).

107. R. Croteau, A. J. Burbott, and W. D. Loomis, Phytochemistry, 11, 2459 (1972).

108. R. Croteau and W. D. Loomis, Phytochemistry, 11, 1005 (1972).

109. M. J. Coon, W. G. Robinson, and B. K. Bachhawat, "Symposiun on Amino Acid Metabolism," Johns Hopkins Press, Baltimore (1955), p. 431.

110. W. G. Robinson, B. K. Bachhawat, and M. J. Coon, J. Biol. Chem., 218, 391 (1956).

111. D. H. G. Crout, J. Chem. Soc., 1233 (1967).

112. E. Leete and J. B. Murrill, Tet. Lett., 1727 (1967).

113. D. H. G. Crout, J. Chem. Soc., 1968 (1966).

114. I. Zubin and K. Bloch, J. Biol. Chem., 185, 117 (1950).

115. A. DelCampello-Campbell, E. E. Dekker, and M. J. Coon, Biochem. Biophys. Acta, 31, 290 (1959).

116. J. Worhmann, H. Yokoyama, and C. O. Chichester, J. Am. Chem. Soc., 79, 4569 (1957).

117. M. J. Coon and S. Gurin, J. Biol. Chem., 180, 1159 (1959).

118. B. K. Bachhawat and M. J. Coon, J. Biol. Chem., 231, 625 (1958).

119. C. O. Chichester, H. Yokoyama, T. O. Nakayama, A. Lukton, and G. Mackinney, J. Biol. Chem., 234, 598 (1959).

120. T. W. Goodwin and W. Lijinsky, Biochem. J., 50, 268 (1952).

121. G. D. Braithwaite and T. W. Goodwin, Biochem. J., 76, 1 (1960).

122. G. D. Braithwaite and T. W. Goodwin, Ibid., p. 5.

123. G. D. Braithwaite and T. W. Goodwin, Ibid., p. 194.

124. J. B. Simpson, Ph.D. Dissertation, Louisiana State University, Baton Rouge, 1979.

125. G. E. Risinger, K. Karimian, S. Jungk, and J. B. Simpson, Experentia, 34, 1121 (1978).

126. K. Karimian, Ph.D. Dissertation, Louisiana State University, Baton Rouge, 1976.

127. G. Mehta, V. R. Nayak, and S. Dev, Tet. Lett., 4561 (1966).

128. G. Popják, D. S. Goodman, J. W. Cornforth, R. H. Cornforth, and R. Ryhage, J. Biol. Chem., 236, 1934 (1961).

129. E. M. Kosower, "Molecular Biology," McGraw-Hill, New York (1962), p. 58.

130. I. Pradip, Ph.D. Dissertation, Louisiana State University, Baton Rouge, 1966.

131. W. H. Schotfer, E. C. Grobb, and G. Bensson, Arch. Sci., Geneva, 5, 5 (1952).

132. M. P. Starr and S. Saperstein, Arch. Biochem. Biophys. 43, 157 (1953).

133. H. D. Durst, M.S. Thesis, Louisiana State University, Baton Rouge, 1966.

134. G. E. Risinger and H. D. Durst, Tet. Lett., 3133 (1968).

135. R. L. Bell, Unpublished results.

136. G. E. Risinger, W. E. Gore, and K. Pulver, Synthesis, 659 (1974).

137. R. R. Williams, J. Am. Chem. Soc., 57, 229 (1935).

138. W. F. Tuley and R. Adams, J. Am. Chem. Soc., 47, 3061 (1925).

139. R. Adams and B. S. Garvey, J. Am. Chem. Soc., 48, 477 (1926).

140. E. C. Horning and M. G. Horning, J. Org. Chem., 11, 95 (1940).

141. L. F. Fieser and M. Fieser, "Reagents for Organic Synthesis," Vol. 1, John Wiley & Sons, New York (1967), p. 46.

142. H. Hirano, K. Morita, Y. Oka, K. Yoshioka, Japan, 67, 18632 (C. A., 69:19200m).

143. J. Metzger, H. Larive, R. Dennilarer, R. Baraller, and C. Gaurat, Bull. Soc. Chim. Fr., 2857 (1964).

144. R. L. Shriner, R. L. Fuson, and D. Y. Curtin, "The Systematic Identification of Organic Compounds," 4th ed., John Wiley & Sons, New York (1948), p. 286.

145. E. Klingsberg and D. Papa, J. Am. Chem. Soc., 73, 4988 (1951).

146. R. L. Shriner, R. C. Fuson, and D. Y. Curtin, Op. cit., p. 200.

147. M. Asano, J. Pharm. Soc. Japan, 480, 97 (1922).

148. L. Ruzicka and G. Firmenich, Helv. Chim. Acta, 22, 392 (1939).

149. J. F. Ruppert and J. D. White, J. Org. Chem., 39, 269 (1974).

150. J. F. Ruppert and J. D. White, Ibid., 41, 500 (1976).

151. L. F. Fieser and M. Fieser, Op. cit., p. 142.

152. M. E. Synerholm, Org. Syn., Coll. Vol. 3, 187 (1955).

153. F. Sondheimer, O. Mancera, M. Urquiza, and G. Rosenkranz, J. Am. Chem. Soc., 77, 4145 (1955).

154. O. Mancera, G. Rosenkranz, and F. Sondheimer, J. Chem. Soc., 2189 (1953).

155. E. P. Papadopolous, A. Jarrou, and C. H. Issidoridies, J. Org. Chem., 37, 615 (1966).

156. R. Radcliff and R. Rodenhorst, J. Org. Chem., 35, 4000 (1970), and references cited therein.

157. K. Bloch, H. Rilling, and O. H. Amdor, J. Am. Chem. Soc., 79, 2646 (1957).

158. T. T. Tchen, "Methods of Enzymology," Vol. 5, Academic Press, New York (1962), p. 489.

159. W. R. Hendee, "Radioactive Isotopes in Biological Research," John Wiley & Sons, New York (1973), p. 186.

160. A. Torriani, "Methods in Enzymology, XII, Nucleic Acids, Part B," Academic Press, New York (1968), p. 212.

161. M. Franch-Neumann and J. J. Lohmann, Tet. Lett., 3729 (1978).

162. P. Grosselin, S. Masson, and A. Thuillier, Tet. Lett., 2717 (1978).

163. O. P. Vig, A. S. Sethi, M. L. Sharma, and S. D. Sharma, Ind. J. Chem., Sect. B, 153, 951 (1977).

164. D. Michelot, G. Linstrumelle, and S. Julia, Synth. Comm., 7, 95 (1977).

165. C. Huynh and S. Julia, Synth. Commun., 7, 103 (1977).

166. D. Michelot, G. Linstrumelle, and S. Julia, Chem. Commun., 10 (1974).

167. J. P. Dillot, J. Dunogues, and R. Calas, Tet. Lett., 1821 (1976).

168. N. P. Damodaran and S. Dev, Tetrahedron, 29, 1209 (1973).

169. A. F. Thomas and W. Pawlak, Helv. Chim. Acta, 54, 1822 (1971).

170. L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," 2nd ed., Pergamon Press, Oxford (1969).

171. Z. Cvengrosova, F. Horak, and M. Hrusovsky, Czech. Pat. 157,929 (C. A., 84:74466a).

172. Z. Cvengrosova, F. Horak, and M. Hrusovsky, Czech. Pat., 156,792 (C. A., 83:43542j).

173. J. B. Clarke and F. J. Pilgrim, J. Am. Chem. Soc., 61, 2667 (1939).

174. H. R. Underwood, Jr. and R. C. Wakeman, J. Am. Chem. Soc., 52, 387 (1930).

175. E. Wedekind and J. Harussermann, Ber., 34, 2081 (1901).

176. L. F. Fieser and M. Fieser, "Organic Chemistry," 3rd ed., Reinhold Publ. Corp., New York (1956).

177. M. W. Rathke, Org. Rxn., 22, 423 (1975).

178. T. Kobayashi, S. Kumazawa, T. Kato, and Y. Kitahara, Chem. Lett., 301 (1975).

179. P. Carré and D. Libermann, C. R. Hebd. Séances Acad. Sci., 199, 1422 (1934).

180. W. R. Brown, J. Chem. Soc., 601 (1945).

181. J. P. E. Homan and J. A. Mills, Nature, 158, 877 (1956).

182. H. H. Bosshard, R. Mory, M. Schmid, and H. Zollinger, Helv. Chim. Acta., 42, 1653 (1959).

183. F. W. McLafferty, ed., "Mass Spectrometry," McGraw-Hill, New York (1963).

184. R. M. Silverstein, G. C. Bassler, and T. C. Morrill, "Spectrometric Identification of Organic Compounds," 3rd ed., J. Wiley & Sons, New York (1974).

185. H. D. Hartzler, J. Am. Chem. Soc., 95, 4379 (1973).

186. H. C. Sorensen and L. L. Ingraham, J. Hetero. Chem., 8, 551 (1971).

187. W. Kirmse, "Carbene Chemistry," 2nd ed., Academic Press, New York (1971).

188. R. Breslow and E. McNelis, J. Am. Chem. Soc., 81, 3080 (1959); 82, 2394 (1960).

189. R. Breslow, Ibid., 80, 3719 (1958).

190. Y. Asahi, M. Nagaoka, Chem. Pharm. Bull., 19, 1017 (1971).

191. H. Vorsanger, Bull. Soc. Chim. Fr., 2124 (1967).

192. J. Hine, J. Am. Chem. Soc., 72, 2438 (1950).

193. J. Hine and A. M. Dowell, Jr., Ibid., 76, 2688 (1954).

194. J. Hine, A. M. Dowell, Jr., and J. E. Singley, Jr., Ibid., 78, 479 (1956).

195. J. Hine, N. W. Burske, M. Hine, and P. B. Langford, Ibid., 79, 1406 (1957).

196. E. D. Bergmann, D. Ginsberg, and D. Lauie, Ibid., 72, 5012 (1950).

197. R. Lombard and R. Boesch, Bull. Soc. Chim. Fr., 733 (1953).

198. J. Hine and P. B. Langford, J. Am. Chem. Soc., 79, 5497 (1957).

199. W. Kautlehner in "The Chemistry of Acid Derivatives, Suppl B," S. Patai, ed., J. Wiley & Sons, New York (1979).

200. G. E. Maier and D. E. Metzler, J. Am. Chem. Soc., 79, 4386 6583 (1957).

201. G. E. Risinger, E. J. Breaux, and H. H. Hsieh, J. Chem. Soc., Chem. Comm., 841 (1968).

202. G. E. Risinger and D. N. Parker, Experentia, 21, 305 (1965).

203. K. Karimian and G. E. Risinger, Org. Prep. Proced. Int., 10, 45 (1978).

204. A. Takamizawa, K. Hirai, Y. Hamashima, and S. Matsumoto, Tet. Lett., 5071 (1967).

205. A. Padwa and P. H. J. Carlsen, Abstr., 176th ACS Natl. Metting, (Orgn.), 87 (1978).

206. K. Karimian, Unpublished results.

207. N. A. Cortese and R. F. Heck, J. Org. Chem., 43, 3985 (1978).

208. P. S. Gradeff, U. S. Pat., 3,971,830 (C. A., 85:124197o).

209. A. Polito, G. Popják, and T. Parker, J. Biol. Chem., 247,

210. K. Ogura, T. Koyama, and S. Seto, J. Am. Chem. Soc., 94, 307 (1972).

211. A. A. Qureshi, F. J. Barnes, E. J. Semmler, and J. W. Porter, J. Biol. Chem., 248, 2755 (1973).

212. S. Mizuhara and P. Handler, J. Am. Chem. Soc., 76, 571 (1954).

213. E. Beytia, A. A. Qureshi, and J. W. Porter, J. Biol. Chem., 237, 62 (1962).

214. P. R. Ortiz de Montellano, J. S. Wei, R. Castillo, C. K. Hsu, and A. S. Bopari, J. Med. Chem., 20, 243 (1977).

215. W. W. Washburn, R. Kow, Tet. Lett., 1555 (1977).

216. P. R. Ortiz de Montellano and A. S. Bopari, Biochem. Biophys. Res. Comm., 76, 520 (1977).

217. P. R. Ortiz de Montellano, J. S. Wei, C. K. Hsu, and A. S. Bopari, J. Med. Chem., 20, 243 (1977).

218. F. Brody and M. T. Bogert, J. Am. Chem. Soc., 65, 1080 (1943).

219. M. B. Doughty, Personal communication.

220. R. Laliberté, H. Warwick, and A. Midway, Can. J. Chem., 46, 3643 (1968).

221. R. H. Wiley, D. C. England, and L. C. Behr, Org. Rxn., 6, 367 (1951).

222. R. S. Egan, J. Tadanier, D. L. Garmaise, and A. P. Gaunce, J. Org. Chem., 33, 4422 (1968).

223. L. S. Rondestvedt, Jr. and J. C. Wygant, J. Am. Chem. Soc., 76, 509 (1954).

224. K. Rühlmann, M. Theil, and F. Asinger, Ann., 622, (1959)

# VITA

Steven J. Jungk was born in Wilmington, Delaware on October 23, 1955. He attended St. Xavier High School in Louisville, Kentucky. After one year of undergraduate training at Pennsylvania State University, he finished his curriculum and received a Bachelor of Science degree from the Louisiana State University in 1976. The author is presently a candidate for the degree of Doctor of Philosophy in Biochemistry.

**EXAMINATION AND THESIS REPORT**

**Candidate:** Steven J. Jungk

**Major Field:** Biochemistry

**Title of Thesis:** On the Biosynthesis of Irregular Terpenes: Mechanistic Studies Employing the Thiamine Thiazole as an Isoprenoid Analogue

Approved:

E. E. Ginger
Major Professor and Chairman

James D. Ingraham
Dean of the Graduate School

EXAMINING COMMITTEE:

R. L. Allen

Elliot S. Jonathan

Thomas H. Fisher

Ronald W. Blakeney

**Date of Examination:**

April 6, 1981